

**OVARIAN RESERVE TESTING IN
PREMENOPAUSAL RECIPIENTS OF
CHEMOTHERAPY FOR BREAST CANCER**

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ABSTRACT

The incidence of breast cancer has progressively increased, while survival rates have simultaneously improved. Young women with breast cancer are likely to suffer ovarian damage from chemotherapy, which can have a profound effect on their quality of life. At present, it is impossible to predict the functional lifespan of the chemotherapeutically damaged ovary as there is insufficient data. Ovarian reserve tests (ORT) have the potential to estimate the reproductive age of the ovaries, which would allow an accurate estimation of fertility status and the risk of premature ovarian failure.

This project investigates the use of ORT in young women with breast cancer. To achieve this, biochemical and biophysical ORT were performed in a mixed longitudinal and cross-sectional study group comprising young women treated with chemotherapy for breast cancer and age-matched, regularly menstruating controls with proven fertility. Overall the results indicate potential for the use of inhibin B, anti-müllerian hormone, oestradiol and antral follicle count in the evaluation of these patients. An *in vitro* study was performed to supplement the clinical study, in which the effects of cytotoxic agents commonly used to treat breast cancer were examined by simultaneously applying equivalent doses of each to a breast cancer cell line and primary granulosa-luteal cell cultures respectively. The effects of these agents were measured in terms of cellular integrity (apoptosis) and functionality (hormones). Overall the results suggest variations in cytotoxicity (LD_{50}) between breast and granulosa cells which have potential therapeutic implications.

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DEDICATION

To my wife Jui,

for encouraging me even during the most difficult of times

and to my children Karanvir, Aishwarya and Vedant,

for loving me despite my absence.

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ABBREVIATIONS

AFC	Antral follicle count
AMH	Anti müllerian hormone
ART	Assisted Reproductive Technologies
b	Basal
BMI	Body mass index
CCCT	Clomiphene citrate challenge test
COCp	Combined oral contraceptive pill
COH	Controlled ovarian hyperstimulation
CMF	Cyclophosphamide, Methotrexate and 5-Fluorouracil
CRA	Chemotherapy related amenorrhoea
E-CMF	Epirubicin, Cyclophosphamide, Methotrexate and 5-Fluorouracil
E ₂	Oestradiol
EFORT	Exogenous FSH stimulation test
ELISA	Enzyme-Linked ImmunoSorbent Assay
ER	Oestrogen receptor
FEC	5-Fluorouracil, Epirubicin and Cyclophosphamide
FSH	Follicle stimulating hormone
G	Gemcitabine
G-test	GnRH agonist stimulation test
GI-PGF	Gonadal insufficiency-premature gonadal failure
GnRHa	Gonadotrophin releasing hormone analogue
hFF	Human follicular fluid
HMG	Human menopausal gonadotrophin

HPO	Hypothalamic-pituitary-ovarian axis
HRT	Hormone replacement therapy
ICSI	Intracytoplasmic sperm injection
IVF	In vitro fertilisation
IVM	In vitro maturation
LH	Luteinising hormone
MAFC	Mean antral follicle count
MOV	Mean ovarian volume
MPI	Mean pulsatility index
MPS	Mean peak systolic velocity
ORT	Ovarian reserve tests
OV	Ovarian volume
OSBF	Ovarian stromal blood flow
OFD	Ovarian follicle density
P	Paclitaxel
PgR	Progesterone receptor
POF	Premature ovarian failure
QOL	Quality of life
RCT	Randomised controlled trial
TAFC	Total antral follicle count
TANGO	Phase III randomised trial of gemcitabine in paclitaxel-containing, epirubicin-based, adjuvant chemotherapy for ER/PgR-poor, early stage breast cancer
TOV	Total ovarian volume
TGF- β	Transforming growth factor β
UCL	University College London

1. BACKGROUND

1.1 BREAST CANCER IN YOUNG WOMEN

1.1.1 INCIDENCE AND SURVIVAL

Breast cancer is the most common cancer in females in England and Wales, with 41,720 new cases diagnosed in 2002 (Office for National Statistics 2005; Welsh Cancer Intelligence and Surveillance Unit. 2005).

Incidence rates have continued their upward trend, increasing by 80 per cent since 1971, and by 16 per cent in the ten years to 2003 (Office for National Statistics 2005). Survival trends have also steadily improved, with the decline in age-adjusted breast cancer mortality rates almost certainly due to improvements in the earlier detection of smaller, palpable tumours and in adjuvant chemotherapy (Jatoi & Miller 2003; Mettlin 1999). This trend in the United Kingdom is also apparent in many other countries in Europe (Botha et al. 2003).

Although very few cases of breast cancer occur in women in their teens or early 20s, breast cancer is the most commonly diagnosed cancer in women under 35 in the UK. By age 35-39 over 1,400 women are diagnosed each year (Cancer Research UK 2006). In the United States, of more than 200,000 new diagnoses of breast cancer made each year, 15% occur in the reproductive years (< 45 years) (Jemal et al. 2004). Furthermore, women are delaying childbearing for domestic, professional and educational reasons (Friedlander & Thewes 2003). In England and Wales the average age of mothers at childbirth has increased by three years since 1971, rising from 26.2 years to 29.1 years in 2000 (Office for National Statistics 2005).

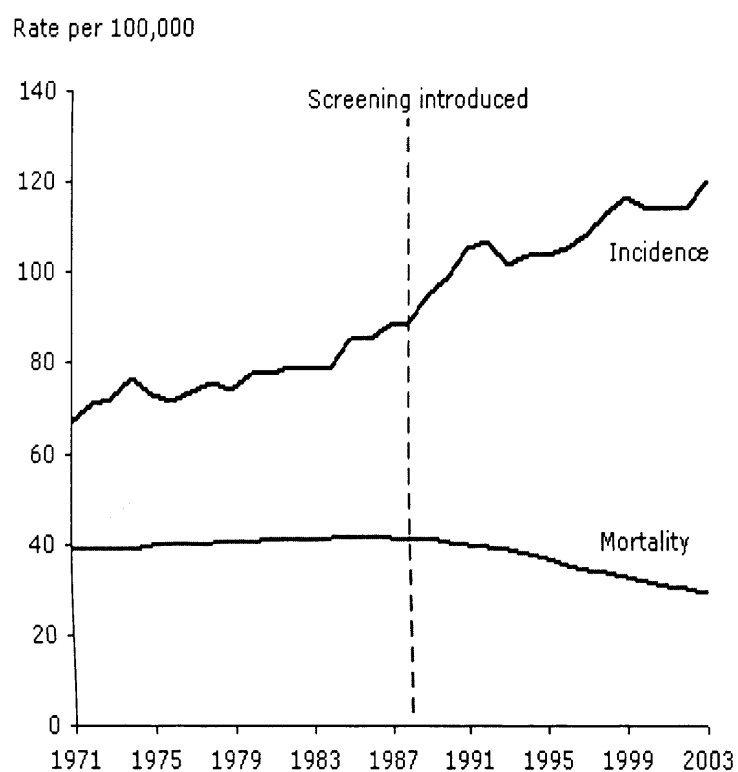


FIGURE 1.1. 1 Age-standardised incidence of and mortality from female breast cancer, England and Wales, Rate per 100,000

Source: National Statistics website: www.statistics.gov.uk

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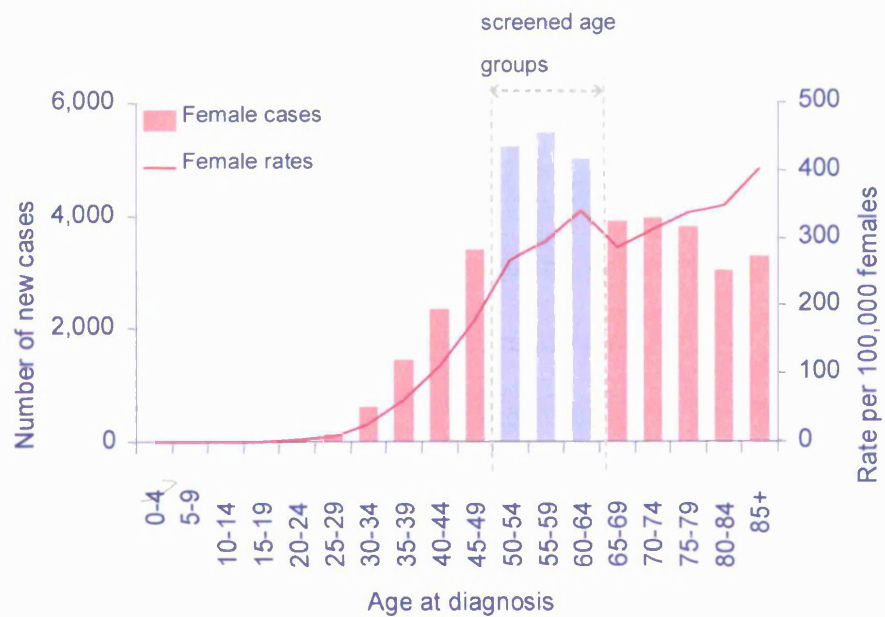


FIGURE 1.1. 2 Number of new cases and age-specific incidence rates for female breast cancer in the United Kingdom 2002.

Source: Cancer Research UK

1.1.2 TREATMENT AND PROGNOSIS

Younger age at diagnosis is considered a poor prognostic feature in breast cancer (Goldhirsch et al. 2001). These women have a greater chance of having an endocrine – unresponsive tumour, and are more likely to present with a higher grade tumour than their postmenopausal counterparts (Colleoni et al. 2002). Compared with older premenopausal women, invasive breast cancer occurring before the age of 35 years has a more aggressive biological behaviour and is associated with a worse prognosis (Shannon & Smith 2003). Those with oestrogen receptor positive tumours are commonly advised to receive ovarian suppression in addition to chemotherapy, as prognosis may be improved (Aebi et al. 2000; Collichio & Pandya 1994; Early Breast Cancer Trialists' Collaborative Group 2000).

Although adjuvant chemotherapy is effective in all age groups, the magnitude of benefit is greatest in younger premenopausal patients (Early Breast Cancer Trialists' Collaborative Group 2002; Gradishar 2003). This has led to the evaluation of more aggressive therapies such as high-dose chemotherapy with haematopoietic stem cell rescue or autologous stem cell transplantation in women with metastatic and early poor prognosis breast cancer (Farquhar et al. 2003b; Farquhar et al. 2003a). This therapy, which one would expect to have significant gonadotoxic effects, does not yet show improved outcome compared to conventional chemotherapy (Tallman et al. 2003).

First-line chemotherapy regimes for breast cancer commonly contain cyclophosphamide (C), either as part of the CMF regimen (M = methotrexate, F = 5-fluorouracil) or are anthracycline based, containing either epirubicin or doxorubicin. Therapy is usually given during 3 – 6 cycles and is individualised. Subtle differences only exist between the 2 regimens (Early Breast Cancer Trialists' Collaborative Group 2002) in terms of survival advantage. Newer drugs are always being added, which is

leading to an increased emphasis on targeted therapy (Smith 2002). Taxanes (Docetaxel and Paclitaxel) are commonly used in metastatic disease, usually in association with an anti-metabolite (e.g. Gemcitabine), and are the subject of ongoing research in treating primary disease (Nabholtz & Gligorov 2005). The newest agents, referred to as targeted biologics (trastuzumab) are currently receiving intense interest (O'Shaughnessy 2005).

Women with breast cancer who are contemplating having a family have additional concerns, given the fact that they are usually advised to delay childbearing for 2 - 5 years following diagnosis owing to the increased likelihood of recurrence within the first few years following treatment (Danforth, Jr. 1991; Velentgas et al. 1999). Furthermore, patients with oestrogen-receptor positive disease are usually treated with adjuvant hormonal therapy such as tamoxifen (Early Breast Cancer Trialists' Collaborative Group 2001), in which case pregnancy is not advised for at least 5 years.

1.2 CHEMOTHERAPY-MEDIATED GONADAL DAMAGE

1.2.1 INTRODUCTION

The gonadotoxic effect of chemotherapeutic agents is well documented, although the prevailing mechanisms are not fully understood.

In general, alkylating agents such as cyclophosphamide (C) which are non-cell cycle specific, are more cytotoxic to the ovaries than cell cycle specific agents such as methotrexate (M) and fluorouracil (F), whose major effect is on ovarian follicle growth and maturation (Hensley & Reichman 1998). This may affect sex steroid production, leading to disturbance of the hypothalamic-pituitary-ovarian (HPO) axis (Dnistrian et al. 1985; Dowsett & Richner 1991; Mehta, Beattie, & Das Gupta 1992). Ovarian biopsies in patients undergoing cyclophosphamide-based treatment reveal complete absence of ova or small numbers of inactive ova with fibrosis and no evidence of follicular maturation (Koyama et al. 1977; Warne et al. 1973). In an animal study, Meirow and colleagues found a significant inverse relationship between the dose of cyclophosphamide and the total number of follicles counted in the ovaries (Meirow et al. 1999).

1.2.2 AMENORRHOEA AND PREMATURE OVARIAN FAILURE

Chemotherapy-related-amenorrhoea (CRA) is a term used to describe the occurrence of amenorrhoea following chemotherapy, the rate of which varies according to the diagnostic criteria used and length of follow-up (Bines, Oleske, & Cobleigh 1996). Its onset is mediated through ovarian failure, and is based on the observation that the hormone profile observed in premenopausal women treated with adjuvant chemotherapy for breast cancer who develop CRA is consistent with primary ovarian failure (Dnistrian et al. 1983; Dowsett & Richner 1991; Rose & Davis 1977). The risk

of POF with multiple agent adjuvant chemotherapy has been reported to range from 53% to 89% (Del Mastro et al. 1997). The rapid evolution of chemotherapeutic regimens for breast cancer however (Awada et al. 2003), limits the usefulness of this information in modern practice.

Generally, the risk of POF is related to the patient's age, treatment protocol and type of malignancy (Meirow & Nugent 2001). Age and time to CRA are inversely related, as well as cumulative dose required to produce CRA. The average incidence of CRA for CMF - based regimens is 40% for women less than 40 years of age and 76% for those older than 40 (Bines, Oleske, & Cobleigh 1996). This may be explained by the reduction of primordial follicles with age (Faddy & Gosden 1996), thus making these women more susceptible to the effects of chemotherapy. A cohort of premenopausal women receiving either adjuvant CMF, CEF (E- Epirubicin), tamoxifen, or no treatment was followed up prospectively for one year (Goodwin et al. 1999). Age and the use of systemic chemotherapy were found to be independent predictors of premature menopause. The use of CMF or CEF, whether in combination or not with tamoxifen, increased the risk of menopause in 40-year-old women from less than 5% to more than 40% (Goodwin et al. 1999). It would appear that anthracycline-containing regimens such as FEC or AC (A=doxorubicin) carry a lower risk of inducing POF compared to classic CMF regimens (Levine et al. 1998; Minton & Munster 2002). Data regarding the effect of taxanes is limited. A recent retrospective study reported that the incidence of CRA (defined here as amenorrhoea persisting for > 12 months after treatment) in a group of women < 40 years of age treated with adjuvant anthracycline and taxane-based chemotherapy was 15% (Fornier et al. 2005). One of the reasons these studies might report lower CRA rates is the fact that the cumulative dose of cyclophosphamide is lower in such regimens. For example AC usually consists of 4 cycles, whereas standard CMF consists of 6 cycles (see figure 2.2.1). It would seem therefore, that the major

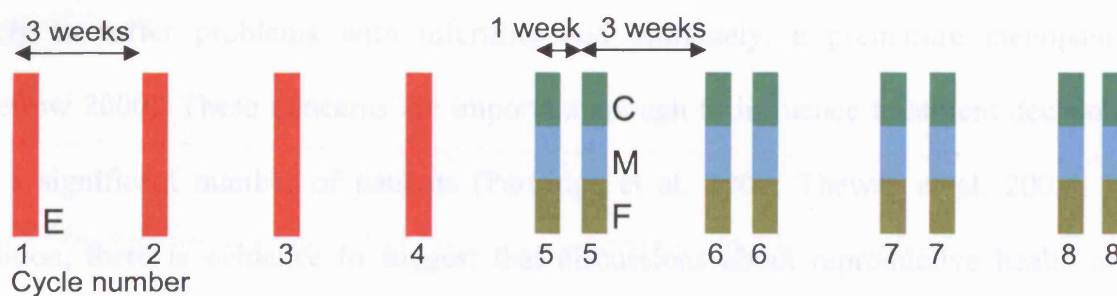
gonadotoxic effect from adjuvant chemotherapy regimes for breast cancer stems from the alkylating agent cyclophosphamide.

There is some data to suggest that commencing chemotherapy during the follicular phase of the menstrual cycle is associated with a higher incidence of CRA (Di Cosimo et al. 2004), a finding not detected previously (Mehta, Beattie, & Das Gupta 1992). The former study was limited by the fact that CRA was defined as cessation of menses for at least 3 months during or after chemotherapy, highlighting the importance of a lack of a unified definition for CRA. The median age was also higher in comparison to other studies (42 years).

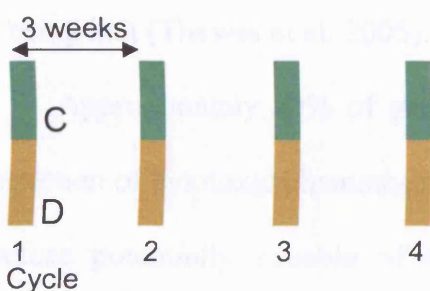
Whereas the induction of ovarian failure may be advantageous with respect to breast cancer outcome (Pagani et al. 1998), it is not clear that there is any advantage to permanent menopause over reversible hormonal manipulations (Moore 2000). Restoration of menstruation after CRA is possible. Again this is influenced by age and duration of follow-up, and has been estimated at 22% to 56% in younger women and 0% to 11% in older patients (Bines, Oleske, & Cobleigh 1996). However, women who maintain normal menses throughout chemotherapy remain at risk for developing POF. This is evident from the high rates of POF seen in adolescents receiving alkylating agents for cancer (Byrne et al. 1992).

Ovarian damage following chemotherapy can present with a variety of symptoms which reflect varying degrees of damage, culminating in premature ovarian failure (POF) (Moore 2000). POF is associated with a wide array of effects including vasomotor symptoms (hot flashes and night sweats), genitourinary symptoms (vaginitis, dyspareunia, dysuria) and may contribute to sexual dysfunction (Bakewell & Volker 2005; Broeckel et al. 2002). Ovarian failure after adjuvant chemotherapy has been shown to be associated with rapid bone loss in women with early-stage breast cancer (Shapiro, Manola, & Leboff 2001) and osteoporosis that can lead to skeletal fractures

(Ganz & Greendale 2001). The management of these symptoms is particularly difficult in patients with breast cancer owing to concerns about hormone replacement therapy (HRT; Beral 2003; Biglia et al. 2003; Rostom 2001).



E-CMF



AC x 4

FIGURE 1.2. 1 Standard chemotherapy schedules for breast cancer

E = Epirubicin, C = Cyclophosphamide, M = Methotrexate, F = 5-Fluorouracil

A = Anthracycline (doxorubicin)

(Kindly provided by Dr Rob Stein).

1.2.3 EFFECT ON FERTILITY

Young breast cancer survivors are very concerned about their reproductive capability (both before and after treatment), which can have a profound impact on their self-esteem (Ganz 2000). Those patients who are not immediately rendered infertile are still likely to suffer problems with infertility and ultimately, a premature menopause (Meirow 2000). These concerns are important enough to influence treatment decisions for a significant number of patients (Partridge et al. 2004; Thewes et al. 2005). In addition, there is evidence to suggest that discussions about reproductive health are overlooked in the counselling process prior to chemotherapy (Duffy, Allen, & Clark 2005), and that the information needs of these patients with regard to these issues are not being met (Thewes et al. 2005).

Approximately 50% of patients younger than 35 resume normal menses after completion of cytotoxic chemotherapy (regardless of cancer type; Forbes 1992), and are therefore potentially capable of becoming pregnant. However, although a regular menstrual cycle may serve as a convenient marker for ovarian function, a normal menstrual cycle is not synonymous with fertility. Similarly, irregular menses or amenorrhoea does not always imply infertility. This was shown in a retrospective series of women who received chemotherapy with FAC (A- anthracycline) who were 35 years or younger at the time of treatment. In this series, 33 pregnancies occurred in 25 patients (21%), of which only 64% continued to menstruate regularly during and after chemotherapy (Sutton, Buzdar, & Hortobagyi 1990).

Because of the possibility of spontaneous recovery, ovarian function should be reassessed periodically in patients with chemotherapy-induced gonadal damage. Conversely, if fertility is not desired, contraception should be used, and the combined

oral contraceptive pill (COCP) has been used for hormone replacement therapy (Nasir et al. 1997).

The potential effect of treatment on fertility in other cancers was illustrated by a large retrospective survey of pregnancy outcomes after peripheral blood or bone marrow transplantation which revealed that only 0.6% of patients conceived after autologous or allogenic stem cell transplantation (SCT), but the pregnancies were likely to have a successful outcome (Salooja et al. 2001).

Overall the data available relating to pregnancy rates and outcomes following or during breast cancer is limited and relates only to small, defined cohorts which may not be representative of the entire population. Furthermore, follow up is almost always limited in duration.

1.3 BREAST CANCER AND PREGNANCY

1.3.1 INTRODUCTION

Women who contemplate childbearing after a diagnosis of breast cancer have to consider (Collichio, Agnello, & Staltzer 1998) the effect pregnancy will have on their cancer, and vice versa. It is important to consider these very difficult issues, which are made even more complex by the fact that their ability to conceive may already be limited.

1.3.2 EFFECT OF PREGNANCY ON DISEASE RECURRENCE

Patients who wish to have children after treatment for primary breast cancer may seek advice as to whether or not pregnancy would increase their chance of disease recurrence. Although it is clear that a woman's risk of developing breast cancer is closely related to reproductive factors, the exact relationship, especially with regards to prognostic influence, is not clearly defined (Daling et al. 2002). It has been estimated that 3-7% of women become pregnant following a diagnosis of breast cancer (Kroman et al. 1997b; Saunders & Baum 1993). Reviews on the topic agree that there is no evidence that pregnancy after breast cancer treatment has a negative influence on prognosis (Kasum 2006; Kroman & Mouridsen 2003; Upponi et al. 2003). The evidence for this is based mainly on retrospective, case-control studies with limited numbers (Dow, Harris, & Roy 1994; Kroman et al. 1997; Mueller et al. 2003; Sankila et al. 1998; Velentgas et al. 1999; von Schoultz et al. 1995). Gelber and colleagues performed a retrospective review where an attempt was made to form a non-pregnant comparison group, matched according to age, nodal status, tumour size and year of diagnosis. Patients in the non-pregnant group were free of relapse for at least as long as the time between breast cancer diagnosis and completion of pregnancy for patients in the pregnant group (Gelber et al. 2001). Overall survival in the pregnant group, as assessed by 5- and 10- year survival percentages was actually superior to the non-pregnant group (Gelber et al 2001). Although this may be explained by a proposed anti-tumour effect of pregnancy, it may also be explained by a previously reported "healthy mother effect" (i.e., that only women who "feel healthy" get pregnant and those who are affected by the disease do not); (Sankila, Heinavaara, & Hakulinen 1994).

Therefore, despite encouraging reports to date, there is a need for prospective data to clarify the issue convincingly (Surbone & Petrek 1997), especially in younger

premenopausal patients who are at a higher risk of relapse (Kroman et al. 2000). The best evidence to address this question is never likely to be forthcoming, as it would be impossible to perform a randomised controlled trial in this context.

1.3.3 TERATOGENICITY

It is known from animal studies that various cytotoxic agents are mutagenic to female germ cells at various stages of maturation (Meirow et al. 2001). Studies in humans have not reported the expected translation of these effects into increased miscarriage rates or congenital abnormalities compared to the general population, but these studies were confined to patients with haematological malignancy (Green 1997; Holmes & Holmes 1978; Sanders et al. 1996). These findings provide reassurance but must be cautiously interpreted due to the lack of data in humans (Meirow & Schiff 2005).

1.4 OPTIONS FOR PRESERVING FERTILITY

1.4.1 INTRODUCTION

Advances in reproductive technology have made fertility preservation techniques a real possibility for these patients. Decision-making in this area is particularly difficult because of the experimental nature of many techniques.

1.4.2 EMBRYO CRYOPRESERVATION

This is the only established method available in clinical practice to preserve fertility in women being treated for cancer prior to chemotherapy (Revel & Schenker 2004). Pregnancy rates are reported to be 20-30 % per transfer of two to three embryos (Lobo 2005). Drawbacks to its use include the need for a partner (or donor sperm), the fact that a harvest of viable embryos cannot be guaranteed, and any complications or delay in the IVF cycle may delay the commencement of chemotherapy (Thomson et al. 2002).

Perhaps the main obstacle to its use in patients with breast cancer involves the need for a stimulated cycle and IVF, which is theoretically harmful to patients with hormone-sensitive breast cancer. This risk has been circumvented in the past by employing natural-cycle IVF (Brown et al. 1996), but pregnancy rates are low with this method (Pelinck et al. 2002). Tamoxifen has been shown to increase ovulation rates in premenopausal women (Sunderland & Osborne 1991). IVF and embryo cryopreservation after ovarian stimulation with tamoxifen has been employed with some success whilst possibly providing a safer alternative to ovarian stimulation in these patients (Oktay et al. 2003). In that study, tamoxifen stimulation resulted in 2.5-fold increase in embryo yield compared to patients undergoing natural-cycle IVF without cancer recurrence in short-term follow-up. Tamoxifen is known to increase oestradiol levels in premenopausal women (Jordan et al. 1991). Based on this knowledge, the same

investigators studied the use of a 3rd generation aromatase inhibitor (Letrozole), which would be expected to induce ovulation without raising oestrogen levels. Combination of Letrozole with FSH stimulation in a prospective cohort of patients with breast cancer yielded a higher number of embryos than tamoxifen stimulation alone, without the associated rise in oestradiol nor an increased recurrence rate (Oktay et al. 2005). These results have been interpreted with caution however, as the study design (small sample, non-randomised short follow-up) precludes any definitive conclusions about the safety of IVF in patients with breast cancer (Partridge & Winer 2005). Finally, the fate of the embryos should be considered at the outset, given the possibility that potential offspring may lose their mothers to the disease (Posada, Kolp, & Garcia 2001).

1.4.3 OOCYTE CRYOPRESERVATION

This is an attractive option for women without a partner. Drawbacks are analogous to embryo cryopreservation as a stimulated cycle is required.

Since the first report of a birth from a frozen oocyte (Chen 1986), the results of this procedure worldwide have been variable with a reported success rate of < 2% despite the improved success rate when combined with ICSI (Porcu et al. 2000). The poor success rates are due to the fragility of the meiotic spindle upon thawing, with an estimated 37% surviving the thawing process (Fabbri et al. 2001). Oktay and colleagues confirmed in a recent meta-analysis that live-birth rates per oocyte thawed were significantly lower (1.9% with a slow-freeze protocol) when compared with the case of IVF with unfrozen oocytes (Oktay et al 2006). Despite this, the technique is still considered to have an important place in reproductive medicine, especially in this group of patients who require fertility preservation (Van der Elst 2003).

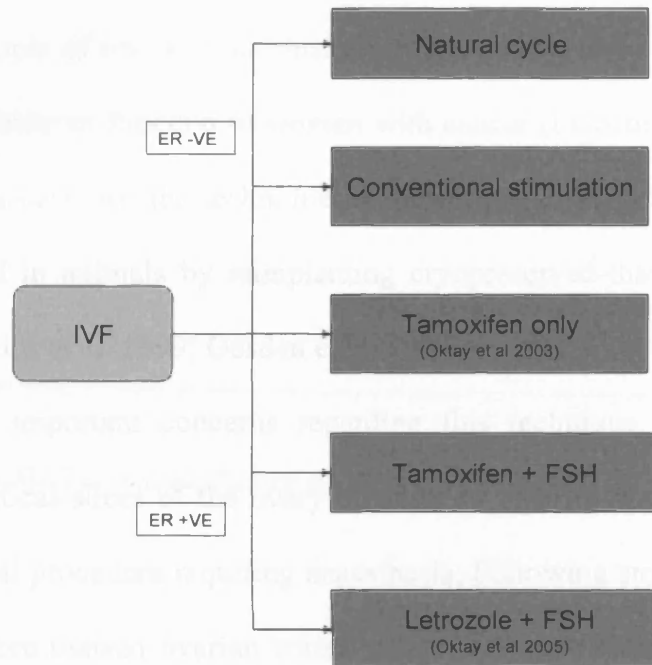


FIGURE 1.4. 1 Options for IVF in patients with breast cancer.

A suggested approach for oestrogen-receptor negative (ER –VE) patients might be to undergo natural-cycle or conventional IVF. Since the possibility exists that ER-VE patients can still have a small fraction of cells staining for the ER, one might consider Tamoxifen or Letrozole stimulated protocols for all patients. If high ER positivity is a concern, one might exclude the Tamoxifen/FSH protocol as this can cause high circulating oestradiol levels (Oktay et al. 2005).

1.4.4 OVARIAN TISSUE CRYOPRESERVATION

This has been a topic of renewed and intense interest as a viable alternative to restore both fertility and ovarian function to women with cancer (Falcone et al. 2004). Earlier studies provided a basis for the technique by showing that pregnancies and livebirths could be achieved in animals by reimplanting cryopreserved-thawed ovarian cortical strips in sheep (Baird et al. 1999; Gosden et al. 1994).

There are important concerns regarding this technique. First, the tissue is obtained from cortical slices of the ovary obtained at laparoscopy, which exposes the patient to a surgical procedure requiring anaesthesia. Following storage, the options are regrafting the frozen-thawed ovarian cortex as an orthotopic (Radford et al. 2001) or heterotopic autograft (Oktay & Karlikaya 2000).

A significant step was achieved when the first livebirth after transplantation of ovarian tissue in non-human primates was reported (Lee et al. 2004). Progress in humans has also finally become possible after several years of effort. Oktay recently described fertilisation and formation of a four-cell embryo using eggs retrieved from re-implanted ovarian tissue from a 30 year old woman with breast cancer (Oktay et al. 2004). In another major breakthrough, Donnez and colleagues recently announced resumption of ovarian function followed by a livebirth in a woman with Hodgkin's lymphoma, who underwent orthotopic autotransplantation of cryopreserved ovarian tissue almost six years after removal (Donnez et al. 2004).

One of the problems encountered with this technique is the short-term viability of the graft, regardless of whether it is an orthotopic or heterotopic allograft, resulting in a limited window of opportunity. This necessitates an intention to conceive within a short space of time, and may preclude patients of an older age group from offering such treatment, as the number of follicles obtained is likely to be lower (Oktay et al. 2001;

Oktaý & Karlikaya 2000). Whole ovary transplantation has been attempted with successful pregnancy following transplantation of frozen-thawed rat ovaries (Wang et al. 2002). An attempt to do the same in humans has recently been reported with limited success (Bedaiwy & Falcone 2004). More recently, using a microsurgical technique, large pieces of ovarian cortex from a monozygotic twin was transferred to the atrophic ovary of a recipient twin who had undergone POF, with viability of the transplant being documented (Silber et al. 2005).

Concern about the possibility of grafting tissue which can potentially harbour malignant cells has been raised using a mouse lymphoma model (Shaw et al. 1996). Recommendations put forward to reduce this risk include the identification of patients with low risk of malignant potential, careful histological evaluation of retrieved tissue and the use of molecular biologic techniques to detect occult cancer cells (Sonmezer, Shamonki, & Oktay 2005). An attractive solution to these concerns would be in vitro maturation of primordial follicles (discussed below) or xenotransplantation. The latter carries the risk of transmission of prions or animal viruses accompanying the retrieved oocytes.

Advances in this field, which have only recently been announced, have created a new sense of enthusiasm and optimism for a technique that up to now has been completely experimental.

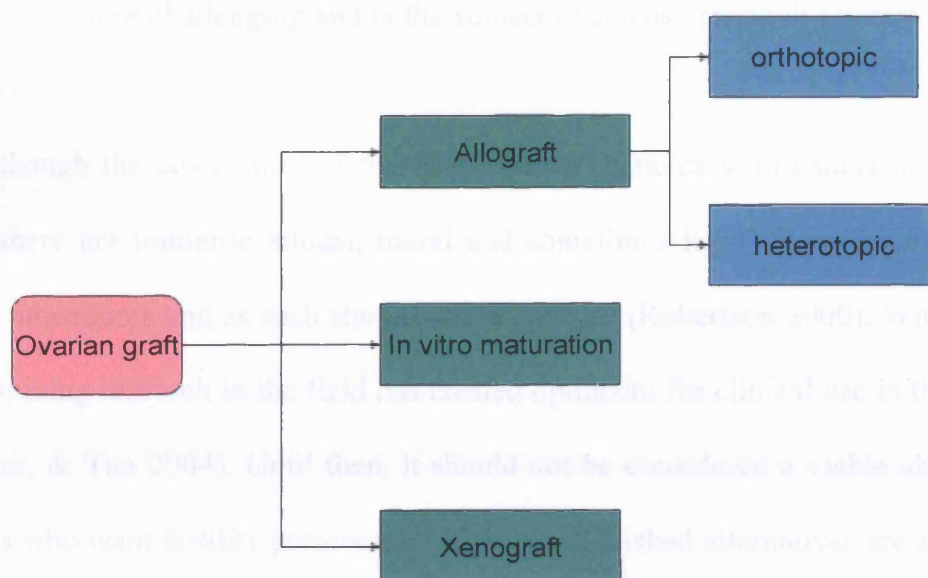


FIGURE 1.4. 2 Options for the use of cryopreserved ovarian cortical tissue.

Ovarian grafts can be re-implanted into the donor (allograft) either at the same site (orthotopic) or elsewhere (heterotopic). Follicles can be cultured from the same tissue to undergo in-vitro maturation (IVM). Here the mature follicles can be cryopreserved for future use. The final option is to xenograft the tissue into an animal host.

1.4.5 IN VITRO MATURATION OF OOCYTES

This is considered an attractive goal as it would eliminate any risk of re-implanting residual cancer cells and could in theory produce more mature oocytes by avoiding follicle wastage created by ischemia or normal atresia. Following retrieval from fresh cortical tissue or follicular aspirate, the oocytes are matured in-vitro following which they may be vitrified or fertilised by ICSI and cryopreserved (Cha & Chian 1998). Although not widely practised, pregnancy rates after IVM (using immature antral follicles) has exceeded 30% in some centres, with an estimated 300 healthy infants born

worldwide (Chian, Lim, & Tan 2004). Primordial follicle culture however has proven to be particularly more challenging and is the subject of intense research interest (Gosden et al. 2002).

Although the advantages of this procedure in patients with cancer are readily apparent, there are immense ethical, moral and sometimes legal dilemmas associated with these procedures and as such should not be ignored (Robertson 2000). With this in mind, continuing research in the field has created optimism for clinical use in the future (Chian, Lim, & Tan 2004). Until then, it should not be considered a viable alternative for patients who want fertility preservation if more established alternatives are an option (Revel & Schenker 2004).

1.4.6 OVARIAN PROTECTION

The potential protective role of luteinizing-releasing-hormone agonists on the primordial follicle pool was first established in rodents (Ataya et al. 1985), then rhesus monkeys (Ataya et al. 1995). Attempts have been made in humans by the administration of prior and concomitant treatment with gonadotrophin releasing hormone analogues (GnRHa) (Blumenfeld et al. 1996; Blumenfeld et al. 1999). These studies were relatively small and the duration of follow up was short, thus preventing a true estimation of the protective effect of GnRH agonists.

Zoladex™ has been compared with CMF where it was found that Goserelin offers an effective, well-tolerated alternative to CMF in premenopausal patients with ER-positive and node-positive early breast cancer (Jonat et al. 2002). Concerns exist regarding the side effects of Goserelin, but these are considered to be reversible (Nystedt et al. 2003).

Despite encouraging results in animal models, few clinical studies have evaluated the effect of GnRHa co-treatment in preventing chemotherapy induced POF in cancer patients (Blumenfeld 2003). The questionable presence of GnRH receptors in human gonadal tissue also challenges the presumed mechanism of action (Clayton & Huhtaniemi 1982). Primordial follicles are believed to initiate growth via an FSH independent receptor mechanism (Gougeon 1998). If this is the case, it implies that GnRH agonists could not mediate their protective effect via FSH receptors. Furthermore, the safety of their use is yet to be established. These questions can only be answered by sufficiently large, randomised prospective studies. One such study is currently underway in the UK attempting to address this issue (**Ovarian Protection Trial In Oestrogen Non-responsive Premenopausal Breast Cancer Patients Receiving Adjuvant or Neo-adjuvant Chemotherapy “OPTION”**; Scottish Cancer Network).

1.4.7 OOCYTE DONATION

It should not go unmentioned that the only option available to women who have undergone POF (other than adoption/surrogacy) remains oocyte donation. In this technique, the donor undergoes IVF while the recipient needs hormone replacement therapy for uterine support. Success rates are very good, in the range of 40 to 50 % per cycle (Lobo 2005). Another advantage is that the age of the recipient is not a factor. One major practical drawback is that donors are very difficult to find.

1.4.8 SUMMARY OF FERTILITY PRESERVATION TECHNIQUES

Fertility preservation techniques have evolved to the extent that there are now several options available to the patient with breast cancer. It is important to remember that despite several encouraging advances made in this area, the best option for patients who are suitable remains embryo cryopreservation. With the continual development of these techniques however, the ideal scenario may develop where it might be possible to offer an individualised approach to management (Del Mastro & Venturini 2006; Sonmezer & Oktay 2006). This process might be facilitated by accurate estimation of a patient's ovarian reserve prior to undergoing fertility preservation, as will be discussed in the following section. Safety remains a major concern however (Gerber et al. 2007; Jones 2006; Wildiers et al. 2006). The American Society of Clinical Oncology (ASCO) has responded to these concerns by publishing guidelines on fertility preservation during cancer therapy (Lee et al. 2006). It is essential therefore that further research is performed to validate the clinical safety of these procedures before making them part of routine clinical practice.

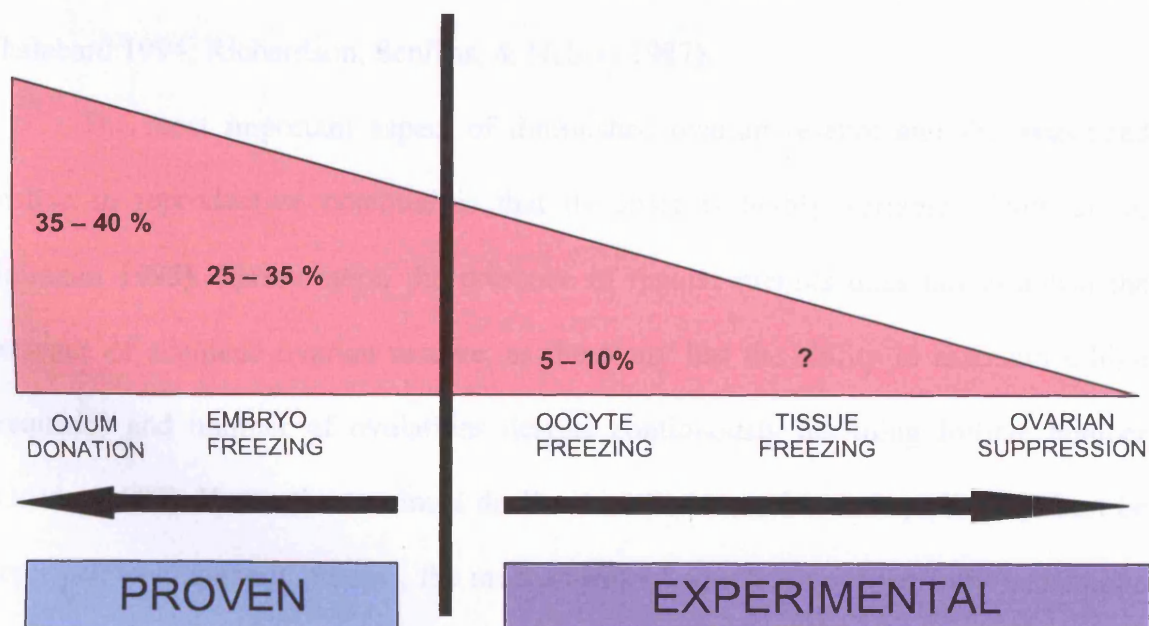


FIGURE 1.4. 3 Success rates for various methods of fertility preservation.

1.5 OVARIAN RESERVE

1.5.1 DEFINITION AND INTRODUCTION

Fertility potential in the female patient is related to the total number and quality of the primordial follicles remaining in the ovaries and is referred to as ovarian reserve. The function of these follicles is twofold: gametogenesis (which governs fertility potential), and steroidogenesis (which governs the onset of a premature menopause).

Ovarian reserve tests (ORT) have become established in the fertility setting where the association of poor ovarian response due to diminished reserve leads to cycle cancellation and reduced success rates in in-vitro fertilisation (IVF) (Pellicer et al. 1987). Although natural fecundity and pregnancy rates decrease with increasing age (te Velde & Pearson 2002), the need for ORT was clearly apparent when it was established that chronological age and menstrual characteristics were unreliable in predicting reproductive age (Scott et al. 1995). Several models have now been described which accurately depict the progressive decline of primordial follicles in the ovary with a woman's age (Faddy et al. 1992; Faddy & Gosden 1996; Gougeon, Ecochard, & Thalabard 1994; Richardson, Senikas, & Nelson 1987).

The most important aspect of diminished ovarian reserve and the associated decline in reproductive potential is that its onset is highly variable (Scott, Jr. & Hofmann 1995). Furthermore, the presence of regular menses does not establish the presence of adequate ovarian reserve, as the ovary has the ability to maintain a high frequency and number of ovulations despite continuously declining follicle number (Gosden 1987). Hence the continual decline in follicle number and quality can best be described as a dynamic process, the mechanisms of which are not yet fully understood (te Velde 1993).

1.5.2 REPRODUCTIVE AGEING AND OVARIAN RESERVE

Reproductive ageing is a concept intimately related to ovarian reserve, as it could potentially predict age at menopause, a measure which is known to be highly variable (Treloar 1981). Biochemical markers also show a marked variability, with FSH levels not increasing permanently until 1-5 years after the onset of the perimenopause (Burger et al. 1999). Inhibins and activins have also been explored (Santoro, Adel, & Skurnick 1999), and it has been suggested that there is a subtle decrease in inhibin B prior to the rise of FSH (Soules, Battaglia, & Klein 1998). Ultrasound determination of ovarian volume and AFC have also been utilised with some success (Flaws et al. 2001).

Several hypotheses have been forwarded in an attempt to describe the process of reproductive ageing (Lobo 2003; Nikolaou & Templeton 2003). The basic doctrine which has prevailed to explain the concept of ovarian reserve and reproductive ageing assumes that the age-dependent loss of fertility in females is dictated by a continual process of follicle depletion (from a pool that is fixed from fetal life), leading to a reduction in both oocyte quantity and quality. This has recently been challenged by the reported finding of the existence of proliferative germ cells that sustain oocyte and follicle production using a mouse model (Johnson et al. 2004b) – a finding that has yet to be replicated or confirmed. The prevailing concept remains therefore that reproductive ageing itself is highly variable (te Velde & Pearson 2002).

1.5.3 OVARIAN RESERVE AND CANCER

The concept of ORT in patients with cancer is a relatively new one, and as such data pertaining to ovarian reserve tests in cancer are limited.

Initial studies in breast cancer documented the effect of cyclophosphamide on cyclical ovarian function (Koyama et al. 1977; Rose & Davis 1977; Warne et al. 1973). Studies at that time were more focused on the therapeutic significance of ovarian suppression in the treatment of breast cancer, and it is fair to say that many clinical trials on breast cancer treatment failed to report this effect on ovarian function. Later studies focused on the relationship between patient age, type and dose of chemotherapy with the onset of amenorrhoea and premature ovarian failure (POF) using clinical and biochemical parameters. Biochemical parameters used were almost exclusively gonadotrophin estimation (FSH and LH) and oestradiol. These studies revealed an endocrine profile in amenorrhoeic patients resembling premature ovarian failure (POF) and an inverse relationship with age (Bonadonna, Rossi, & Valagussa 1985; Dnistrian et al. 1983; Jordan, Fritz, & Tormey 1987; Padmanabhan et al. 1987; Rose & Davis 1977; Samaan et al. 1978; Sherman, Wallace, & Bean 1982). Reduced menstrual activity (not amenorrhoea) was also shown to be associated with a highly variable endocrine profile (Dowsett & Richner 1991). These studies were mostly retrospective and heterogeneous in terms of definitions used for amenorrhoea and POF respectively. They did however establish that ovarian dysfunction was related to patient age, dose and duration of treatment (Reichman & Green 1994; Shapiro & Recht 1994). Ovarian volume has been estimated in patients with breast cancer but the objective here was to establish a reference range for ovarian volume in postmenopausal patients on tamoxifen (Cohen et al. 1997). Overall, there is a lack of pertinent data relating to ovarian reserve testing in patients with breast cancer.

More progress has been achieved with regards to estimating ovarian reserve in patients with haematological malignancy. For example, it has been shown in a prospective study that ovarian volume in patients with lymphoma and leukaemia treated by high-dose chemotherapy and radiotherapy was reduced by 50% within 72 hours of cessation of high dose therapy (Chatterjee et al. 1994). In another study using the HMG test there was diminished ovarian reserve in patients with lymphoma treated by BEAM (carmustine, etoposide, cytarabine and melphalan) chemotherapy, and many patients had diminished ovarian reserve even prior to chemotherapy due to antecedent chemotherapy or disease (Chatterjee & Goldstone 1996). This led to the description of the Gonadal Insufficiency – Premature Gonadal Failure syndrome (Chatterjee & Kottaridis 2002). As the name implies, the syndrome describes a heterogeneous picture whereby the gonadotoxic effect of these agents is not “all or nothing” but can be both acute and cumulative, with the ovaries possessing a limited capacity for recovery. These authors made an attempt to grade the extent of damage using clinical, biochemical and biophysical parameters, however it was noted that the clinical picture was variable and as such did not always comply with these parameters. As such the potential for ovarian recovery, as well as the response to therapy, was unpredictable. While this model may not be directly applicable to breast cancer, it remains an attractive one which highlights the importance of developing tests which can accurately estimate ovarian damage and correlate it with clinical features.

More recently, inhibin A and inhibin B (in addition to FSH, LH and oestradiol), have been assessed prospectively as prognostic factors for resumption of ovarian function in patients with lymphoma who received GnRH analogue co-treatment, with encouraging results (Blumenfeld 2002).

Recognition of the fact that successful treatment of childhood cancer can be associated with impaired gonadal function in adulthood (Thomson et al. 2002) has led to

increased efforts to estimate ovarian reserve in long-term survivors of childhood cancer. Basal gonadotrophins are unreliable in prepubertal children due to the relative quiescence of the HPO axis. Some investigators have proposed the use of inhibin B levels where suppression may have been indicative of arrested follicle development (Crofton et al. 2003). Another study revealed that despite regular menstrual cycles, survivors of childhood cancer had smaller OV, reduced AFC and inhibin B compared with controls (Larsen et al. 2003b). The same authors showed that in a cancer survivor cohort with FSH levels < 10 IU/l, ovarian reserve was still reduced as evidenced by smaller ovarian volumes and lower AFC compared with age-matched controls (Larsen et al. 2003a). A similar cohort of cancer survivors, that included some women on the combined oral contraceptive pill (COCP), had tests of ovarian reserve performed following an FSH stimulation test. Here the investigators found differences in AMH, FSH and OV compared with controls (Bath et al. 2003).

Despite these findings, the data cannot necessarily be extrapolated to patients with different types of cancer due to differences in disease, type of chemotherapy and the demographics of the population studied.

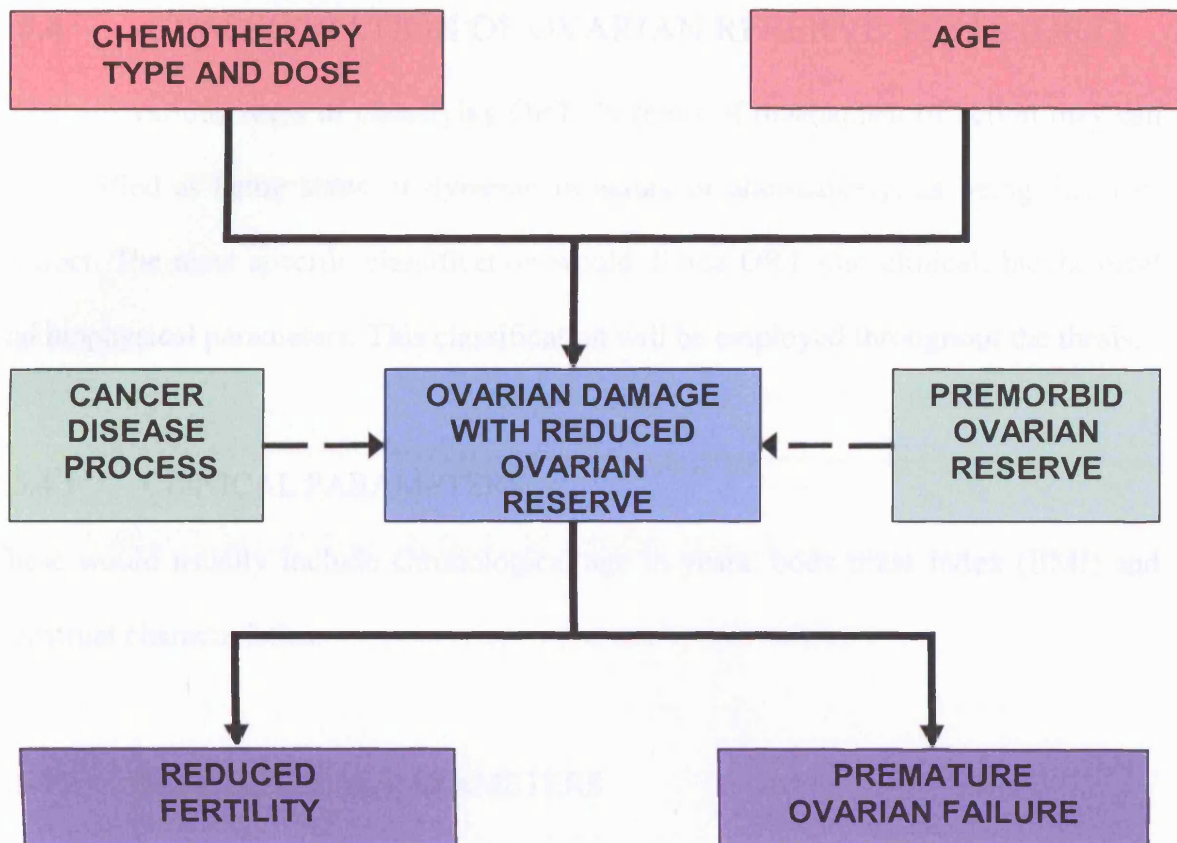


FIGURE 1.5. 1 Pathophysiology of chemotherapy-induced gonadal damage.

Current evidence suggests chemotherapy type and dose in combination with patient age are major determinants of reduced ovarian reserve. Less evidence exists to support the notion that the cancer disease process itself may be implicated, as well as the patient's ovarian reserve prior to treatment. The major sequelae are reduced fertility and premature ovarian failure.

1.5.4 CLASSIFICATION OF OVARIAN RESERVE TESTS (ORT)

There are various ways of classifying ORT. In terms of mechanism of action they can be classified as being static or dynamic in nature or alternatively, as being direct or indirect. The most specific classification would divide ORT into clinical, biochemical and biophysical parameters. This classification will be employed throughout the thesis.

1.5.4.1 CLINICAL PARAMETERS

These would usually include chronological age in years, body mass index (BMI) and menstrual characteristics.

1.5.4.2 BIOCHEMICAL PARAMETERS

These would include basal (early follicular phase) levels of follicle stimulating hormone (FSH), Luteinising hormone (LH), Oestradiol (E2), inhibin A and B, activin A, follistatin and anti-müllerian hormone (AMH). Ovarian stimulation tests include the G-test, the HMG stimulation test, the exogenous FSH stimulation test, and the clomiphene citrate challenge test.

1.5.4.3 BIOPHYSICAL PARAMETERS

These include ultrasound determination of ovarian volume, antral follicle count, doppler indices of ovarian stromal blood flow and uterine dimensions. Also included in this category is histological determination of follicle density in ovarian cortical specimens.

1.5.4.4 RESPONSE TO CONTROLLED OVARIAN HYPERSTIMULATION

This is the response, measured in biochemical, biophysical or other parameters (oocyte or embryo retrieval, pregnancy etc.) to controlled ovarian hyperstimulation (COH) during IVF.

BIOPHYSICAL	BIOCHEMICAL
<p>ULTRASOUND:</p> <ul style="list-style-type: none"> • 2D OR 3D • OVARIAN VOLUME • ANTRAL FOLLICLE COUNT • OVARIAN STROMAL BLOOD FLOW 	<p>BASAL (EARLY FOLLICULAR) LEVELS:</p> <ul style="list-style-type: none"> • FSH, (LH) • E₂ • INHIBIN B, (INHIBIN A) • ANTI MÜLLERIAN HORMONE • (ACTIVIN A, FOLLISTATIN) • (PRO αC)
(FOLLICLE DENSITY)	<p>OVARIAN STIMULATION TESTS:</p> <ul style="list-style-type: none"> • G-TEST • HMG TEST • CLOMIPHENE CITRATE CHALLENGE TEST (CCCT) • FSH (EFORT)

TABLE 1.5. 1 Established biochemical and biophysical ovarian reserve tests in reproductive medicine. Markers in parentheses have as yet a very limited research base to support their use as markers of ovarian reserve.

1.5.5 CLINICAL PARAMETERS

1.5.5.1 CHRONOLOGICAL AGE

While it is important to make the distinction between chronological age as opposed to reproductive age, there is no doubt that the former is inherently linked to ovarian reserve. The evidence pertaining to the continual decline of primordial follicles with increasing age has already been mentioned. Similarly, the effect of age on female fecundity has been well documented (Noord-Zaadstra et al. 1991; Schwartz & Mayaux 1982), while the effect on oocyte quality is depicted in rising aneuploidy rates and implantation failure (O'Connor, Holman, & Wood 1998). Ovarian reserve is not solely dependent on age however. While it is clear that assisted conception outcomes are compromised by advanced maternal age (Klipstein et al. 2005; Swanton & Child 2005), it is certainly not the only factor as outcomes have been shown to be compromised independent of age (Scott et al. 1995). The limitations of chronological age as a marker of ovarian reserve make the investigation of other markers compulsory.

1.5.5.2 BODY MASS INDEX (BMI)

This is an internationally agreed objective assessment of obesity. The exact relationship however between BMI and pregnancy outcome remains unclear. Whilst it seems BMI is related to menstrual irregularity and anovulation, published studies conflict as to whether or not BMI may predict pregnancy outcome (Frankel & Staeheli 1992; Frattarelli & Kodama 2004; Lashen et al. 1999; Lashen, Fear, & Sturdee 2004; Lewis et al. 1990; Loveland et al. 2001; Tinkanen et al. 1999; Wang, Davies, & Norman 2000; Wittemer et al. 2000). It is considered a poor estimate of ovarian reserve.

1.5.6 BASAL BIOCHEMICAL PARAMETERS

1.5.6.1 FOLLICLE STIMULATING HORMONE (FSH)

Basal serum follicle stimulating hormone (bFSH) is considered an indirect estimate of ovarian reserve and depends on the presence of an intact hypothalamic-pituitary-ovarian (HPO) axis. It is one of the longest established parameters for estimating ovarian reserve. The first reports on the usefulness of bFSH measurements showed FSH to be predictive of ovarian response and pregnancy outcome in IVF cycles (Muasher et al. 1988; Scott et al. 1989), and to be more predictive than the patient's age (Toner et al. 1991). Subsequent studies have supported this, including a large retrospective analysis of IVF patients where elevated FSH was associated with high pregnancy loss and poor live birth rates, regardless of age (Levi et al. 2001).

However there are now several studies that challenge the predictive capabilities of bFSH, especially when a patient's age is considered. For example, in patients undergoing their first IVF cycle, bFSH concentration was a better predictor of cancellation rate than age, yet age was a stronger predictor of pregnancy rate (Creus et al. 2000; Sharif et al. 1998). Some studies suggest that young age exerts a protective effect over the deleterious effects of reduced ovarian reserve (Hanoch et al. 1998), while others reveal no difference in outcome between younger and older women with elevated bFSH (El Toukhy et al. 2002). A retrospective cohort study of IVF patients revealed that increasing age, but not bFSH, was associated significantly with reduced implantation and pregnancy rates (Chuang et al. 2003). A recent prospective study in which regularly cycling women above and below the age of 40, with elevated bFSH levels undergoing their first IVF cycle were compared for ovarian response, ongoing pregnancy rates and implantation rates. Although younger women with elevated bFSH levels had higher cycle cancellation rates, implantation and ongoing pregnancy was

superior to their older counterparts (van Rooij et al. 2003). This has prompted the distinction that when a comparison is made, age better reflects egg quality whilst FSH reflects egg quantity (Toner 2003). This study has been criticised however for a lack of adequate controlling and statistical power (McDonough 2003).

Cut-off levels for bFSH are also an issue. In one study, which is unique for its duration of follow-up, ongoing pregnancy was seen in as many as 28% of regularly cycling women with FSH levels between 15-20 IU/l. Only when the FSH level exceeded 20IU/l was there a clear fall in ongoing pregnancy rate regardless of age (van Rooij et al. 2004). A high cut-off level of FSH to achieve acceptable prediction of treatment failure has been reported elsewhere (Bancsi et al. 2000). It seems therefore that younger women with moderate elevations of FSH should not be ignored (Toner 2004). Younger women (age < 41 years) with elevated bFSH levels who have a poor 1st response to IVF however may represent a specific group which should be counselled against further attempts as was shown in a recent retrospective study (Klinkert et al. 2004). Which threshold level of bFSH to use is the question, as some investigators have shown that moderately elevated levels of FSH (defined as 10-11.4 IU/L) are difficult to interpret as these may be confounded by a poor response to gonadotrophin stimulation (Esposito, Coutifaris, & Barnhart 2002).

As most of the data regarding the performance of bFSH in predicting ovarian reserve comes from studies in an IVF population, it is significant that a recent meta-analysis concluded that the performance of basal FSH for predicting poor ovarian response in this group was moderate, while prediction of non-pregnancy was poor (Bancsi et al. 2003). Information pertaining to the general subfertile population is lacking, but a nested case-control study revealed no statistically significant difference in cumulative pregnancy rates between patients with elevated bFSH levels and controls (Van Montfrans et al. 2000). Others have shown elevated bFSH to be of limited value in

patients with regular cycles, in that it should not lead to exclusion of treatment (van Rooij et al. 2004).

Questions remain regarding the reproducibility of bFSH measurements (Sharara, Scott, Jr., & Seifer 1998). One of its main limitations is the significant intercycle variability of FSH, hence limiting the usefulness of a normal value (Scott, Jr. et al. 1990). A recent randomised prospective study revealed that women with limited ovarian reserve exhibited strong intercycle variability of bFSH and FSH response to clomiphene citrate (Kwee et al. 2004). A study performed on normally ovulating women under the age of 35 revealed a large inter-individual variation in bFSH (Schipper, de Jong, & Fauser 1998). Intracycle variability seems to be less of an issue, with some flexibility being apparent in sampling from days 2-5 of a regular menstrual cycle (Hansen et al. 1996; Klein et al. 1996). Repeat basal FSH measurements have also been shown to be futile both in a retrospective (Abdalla & Thum 2006) as well as in a prospective analysis (Bancsi et al. 2004), in that reproductive outcome is not altered.

Another concern is the establishment of normative data within individual laboratories owing to the likelihood of interassay variability (Hershlag et al. 1992). The establishment of threshold values within individual assay systems is also important to prevent errors in interpretation of results especially when clinical outcomes are being correlated. This is more likely to happen in institutions where the clinical volume of patients is insufficient to allow these threshold values to be established (Scott, Jr. 2004).

Other reasons for an elevated bFSH should be considered (Lambalk 2003). Laboratory errors can occur in the presence of heterophilic antibodies, which can interfere with the FSH immunoassay (Cahill, Fox, & Thomas 1992; de Koning et al. 2000). Increased levels and pulsatility of FSH in the follicular phase is seen in mothers of hereditary dizygotic twins (Lambalk et al. 1998). FSH receptor variants have now been identified (Perez et al. 2000; Sudo et al. 2002). There are two known

polymorphisms of clinical relevance in the follicle-stimulating hormone (FSH) receptor exon 10 alanine or threonine at position 307, and asparagine or serine at position 680, giving rise to two discrete allelic variants: Thr307/Asn680 and Ala307/Ser680. It has been hypothesized that FSH receptor genotyping could potentially be used as an adjunct indicator of ovarian reserve as the presence of such variants may cause slightly diminished receptor function, resulting in higher levels of FSH to achieve adequate receptor response whilst not necessarily affecting ovarian reserve (Falconer et al. 2005).

The sensitivity of bFSH in identifying women who will not become pregnant with IVF has been calculated to be only 8%, whereas it is considered to be a specific test, in that 98% of women who achieved pregnancy had normal findings (Barnhart & Osheroff 1998). The positive predictive value (possibility of not becoming pregnant given a positive test result) of the test is difficult to interpret, especially in younger patients or the general subfertile population where the prevalence of nonpregnancy is lower. As such there is a danger of overinterpreting its predictive value (Barnhart & Osheroff 1999). The reasons for these conflicting reports are not entirely clear, but are certainly related to the varying methodologies employed by different investigators, especially with regard to different cut-off values for FSH, study groups, outcome measures and their definitions, duration of follow up and data analysis.

Efforts to increase the sensitivity of prediction have been attempted by combining FSH with Luteinising hormone (LH) as a ratio of FSH: LH with limited success (Kim et al. 1997; Mukherjee et al. 1996).

Despite these limitations, more studies have been performed, and hence much more is known, about the predictive value of FSH than any other marker of ovarian reserve. This, combined with the relative practicality, patient tolerability and low cost of performing the test, is likely to result in basal FSH remaining one of the most commonly performed tests of ovarian reserve for some time to come.

REFERENCE	Scott et al. 1989	Bancsi et al. 2000	Levi et al. 2001	Van Montfrans et al. 2000	Van Rooij et al. 2003	Van Rooij et al. 2004
POPULATION	Assisted reproduction	Assisted reproduction	General subfertile	General subfertile	Assisted reproduction	General subfertile
STUDY TYPE	Retrospective	Retrospective	Retrospective	Nested case-control	Prospective	Retrospective
PT/CYCLE NO.	441/758	435	9802	100	86	122
CUT-OFF (IU/L)	25.0	15.0	14.2	10.0	15.0	15.0
OUTCOME MEASURE	PR	PR	PLR, LBR	PR	PR, IR	PR
CONCLUSION	bFSH predictive of pregnancy outcome	bFSH of limited value in predicting ongoing PR	bFSH predictive of pregnancy outcome	bFSH not predictive of pregnancy outcome	bFSH can discriminate outcome depending on age	bFSH of limited value in predicting outcome

TABLE 1.5. 2 Basal FSH as a predictor of ovarian reserve.

PR = Ongoing pregnancy rate. LBR = live birth rate, IR = implantation rate

1.5.6.2 OESTRADIOL

The condensed follicular phase length in older women may be as a result of a more advanced follicular recruitment by cycle day 3. This early dominant follicle selection is expressed by high serum oestradiol (E_2) concentrations (Licciardi, Liu, & Rosenwaks 1995).

It has been shown in an ART population (where GnRHa's were not administered) that increasing day 3 oestradiol concentrations are associated with decreasing oocyte numbers and pregnancy rates (Licciardi, Liu, & Rosenwaks 1995), a correlation which has been repeated elsewhere (Smotrich et al. 1995). In patients with normal FSH levels, bE_2 has been shown to predict high cancellation rates and low oocyte yield in IVF (Evers et al. 1998). In another study, cancellation rates did correlate with bE_2 levels but did not correlate with pregnancy outcome in those patients who were not cancelled (Frattarelli et al. 2000). Pregnancy rates have also been shown to be higher in a group of women undergoing in-vitro maturation due to male/tubal factor (Mikkelsen et al. 2001). The predictive ability of bE_2 is improved in patients of advanced reproductive age, especially when combined with basal FSH (Buyalos, Daneshmand, & Brzechffa 1997). However, these observations have not been confirmed by others (Lee et al. 1988; Scott et al. 1989). Combination with serum progesterone as a ratio did not improve predictive capacity (Hofmann, Khoury, & Michener 2002; Younis et al. 2001).

No data are currently available regarding basal oestradiol levels in the general subfertile population (Bukman & Heineman 2001). Furthermore there appears to be significant intra-cycle variability (Hansen et al. 1996). Further studies including data on day 3 oestradiol values and fecundity in spontaneous cycles are required before evaluating this parameter further. For the moment it appears not to provide any more information than bFSH alone.

REFERENCE	Licciardi et al. 1995	Smotrich et al. 1995	Buyalos et al. 1997	Evers et al. 1998	Frattarelli et al. 2000
POPULATION	ART	ART	ART	ART	ART
STUDY DESIGN	Prospective	Prospective	Prospective	Prospective cohort	Retrospective
PT/CYCLES	452	225/292	119/380	231	2476
UPPER CUT- OFF (pg/ml)	75.0	80.0	80.0	60.0	80.0
OUTCOME MEASURE	PR	PR, CR	CPR, LBR	Age, RO, FR, CPR	RO, PR, CR
CONCLUSION	Ongoing PR decreases with increasing bE ₂	Increased bE ₂ can predict high CR and low PR independent of bFSH	bE ₂ can predict pregnancy outcome better than age and bFSH	bE ₂ can predict response to IVF in presence of normal bFSH	bE ₂ predicts CR but not pregnancy outcome if ongoing pregnancy

TABLE 1.5. 3 Basal oestradiol as a predictor of fertility.

PR = pregnancy rate, CR = cancellation rate, CPR = clinical pregnancy rate, FR = fertilisation rate,

LBR = live birth rate, RO = retrieved oocytes, ART = Assisted reproduction

1.5.6.3 INHIBINS, ACTIVINS AND FOLLISTATINS

Inhibins and activins are glycoproteins that belong to the transforming growth factor - β (TGF- β) family (see figure 2.5.3). Activins are dimers of β subunits and act as functional antagonists of inhibin to stimulate pituitary FSH synthesis and secretion (Muttukrishna & Knight 1991). Pro α C consists of higher molecular weight inhibins containing the pro region of the α subunit and the biologically inert monomeric pro alpha subunits. Follistatin is a cysteine rich monomeric peptide structurally unrelated to inhibin or activin. Its main function is to bind irreversibly to and neutralize activin (Nakamura et al. 1990). Activins and inhibins and their receptors are known to modulate a variety of physiologic processes in various tissues including the human breast, where they potentially play a role as inhibitors of cell proliferation and breast tumour suppression respectively (Reis et al. 2004).

The role of activin and follistatin as markers of ovarian reserve is not clear. There is little doubt that these hormones have autocrine/paracrine actions on the HPO axis, as shown in patients with PCOS with follicular arrest (Eldar-Geva et al. 2001). More recently, inhibin concentrations from follicular fluid were also shown to be reduced in patients with PCOS (Welt et al. 2005). Overall, it seems from studies on different species that in normal circumstances activin secretion is decreased whilst inhibin and follistatin production is increased as a follicle matures (Schneyer et al. 2000). There is however evidence relating to the role of activin A and follistatin in abnormal pregnancies, an active area of research (Muttukrishna et al. 2004). Most of the following discussion will focus on the role of inhibins as ovarian reserve markers.

Inhibins are heterodimers consisting of two dissimilar subunits (α and β) linked by disulphide bridges, and are secreted by the ovarian granulosa and luteal cells during the menstrual cycle (Lockwood, Muttukrishna, & Ledger 1998). They are a part of the

HPO axis, specifically having an inhibitory effect on pituitary FSH synthesis and secretion (Muttukrishna & Knight 1990) (See figure 2.5.5). Serum dimeric inhibin B is regarded as a direct measure of ovarian reserve as it is mainly secreted by pre-antral follicles (Klein et al. 1996), whereas inhibin A is produced primarily during the late follicular phase by the mature follicle and by the corpus luteum (Roberts et al. 1993).

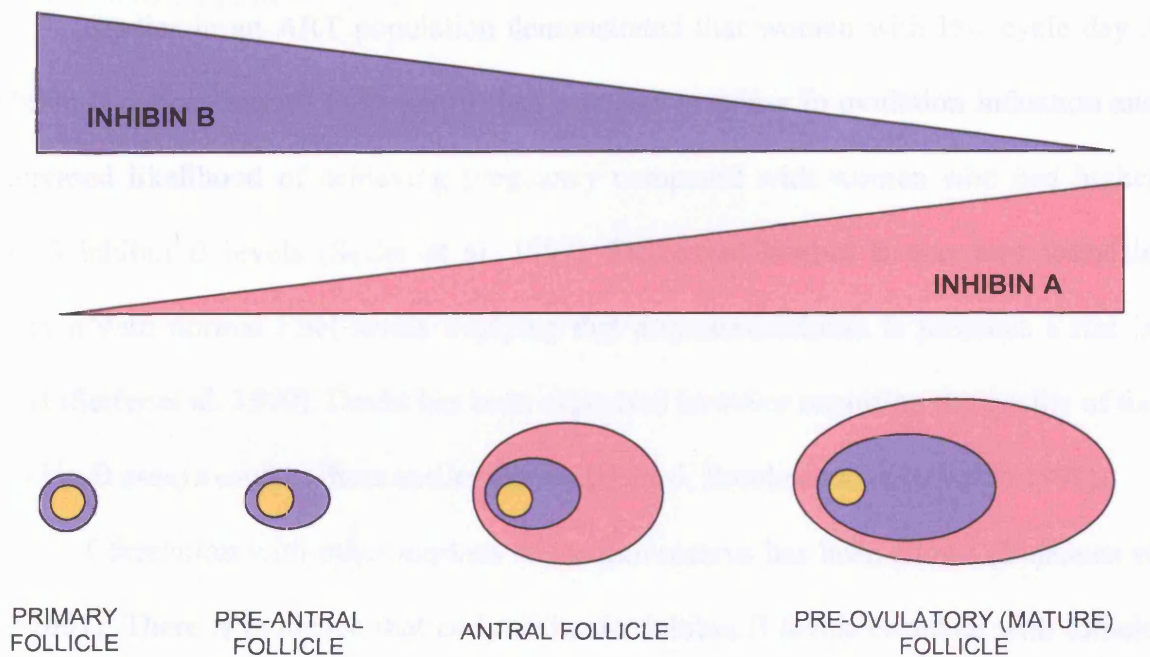


FIGURE 2.5. 2 Differential secretion of inhibin A and inhibin B by granulosa cells at different stages of follicle maturation.

Inhibin B rises from early in the follicular phase to reach a peak coincident with the onset of the mid-follicular phase decline in FSH concentrations, and then declines during the luteal phase apart from a peri-ovular peak, which may represent release of follicular inhibin B from the rupturing follicle into the circulation (Groome et al. 1996; Muttukrishna et al. 1994). Inhibin B has also been shown to respond to exogenous FSH administration in the follicular phase (Burger, Groome, & Robertson 1998). The close temporal relationship between changes in levels of inhibin B and FSH in the mid-follicular phase suggest that the release of inhibin B by the preovulatory follicle

critically regulates pituitary FSH secretion, with inhibin B exhibiting a distinct periodicity in normal women (Lockwood et al. 1998). Studies in older women reveal significantly lower levels of inhibin B in women of 40-50 years of age with raised FSH compared to women with normal FSH (Klein et al. 1996b; Muttukrishna et al. 2000). Additionally, concentrations of inhibin A and B have been shown to be lower in women with elevated FSH and regular menstrual cycles (de Koning et al. 2000).

Studies in an ART population demonstrated that women with low cycle day 3 inhibin B concentrations (<45 pg/ml) had a poorer response to ovulation induction and decreased likelihood of achieving pregnancy compared with women who had higher day 3 inhibin B levels (Seifer et al. 1997). Decreased inhibin B was also found in women with normal FSH levels implying that decreased inhibin B precedes a rise in FSH (Seifer et al. 1999). Doubt has been expressed however regarding the quality of the inhibin B assays used in these earlier studies (Bancsi, Broekmans, & te Velde 1997).

Correlation with other markers of ovarian reserve has been shown (Tinkanen et al. 2001). There is evidence that early follicular inhibin B levels correlate with follicle cohort size (Elting et al. 2001) and oocytes retrieved following controlled ovarian stimulation with FSH (Eldar-Geva et al. 2000; Eldar-Geva et al. 2002), and with an improved sensitivity and specificity compared to other basal markers of ovarian reserve (Ficicioglu et al. 2003). Another study which also showed good correlation with oocytes retrieved did not show a correlation with pregnancy (Fried et al. 2003). Inhibin B levels on cycle day-5 have been shown to be predictive as an early indicator of response during ovarian stimulation as well as outcome (Fawzy et al. 2002; Penarrubia et al. 2000). The predictive ability of basal inhibin B with regards to pregnancy has not been confirmed by several authors, leading to doubt regarding the clinical usefulness of day 3 inhibin levels in clinical practice (Corson et al. 1999; Creus et al. 2000; Dumesic et al. 2001; Hall, Welt, & Cramer 1999).

Studies regarding basal inhibin A have also shown a link with ovarian function and follicular development, but limited value in predicting IVF outcome (Casper et al. 2001; Hall, Welt, & Cramer 1999). Inhibin A may be of more value in older women where it has been shown that the follicular phase peak of inhibin A was significantly lower in women with raised FSH (Muttukrishna et al. 2000).

With regards to dynamic testing, inhibin B has been evaluated in predicting response to ovarian stimulation (Ficicioglu et al. 2003). These include the clomiphene citrate challenge test (CCCT) (Hofmann, Danforth, & Seifer 1998), gonadotrophin-releasing hormone agonist test (G- test) (Ravhon et al. 2000) and the FSH stimulation test (EFORT) (Dzik et al. 2000; Eldar-Geva et al. 2002). These studies are all limited by the fact that although they show correlation with ovarian response, predictive value was not evaluated (Welt 2002). More recently however, studies suggest that the incremental increase in inhibin B following ovarian stimulation may be more predictive of ART outcome than basal levels of inhibin B. In a prospective study, the inhibin B response to exogenous FSH correlated well with the antral follicle count (AFC) and the number of oocytes received (Yong et al. 2003). Kwee and colleagues undertook a prospective randomised study, which compared endocrine markers of ovarian reserve using the CCCT and EFORT to predict ovarian response, and revealed a high correlation with inhibin B in EFORT for number of follicles after ovarian stimulation, with increments in oestradiol and inhibin B providing prediction of ovarian capacity (Kwee et al. 2003).

The reasons for these conflicting reports are not clear and may reflect inter-assay variability and differing reference points and standards amongst different institutions, resulting in reduced specificity of the inhibin assay. Given the expense of performing the test, further prospective evaluation and confirmation of its predictive capacity is required before inhibin B can assume a routine place in the clinical assessment of women with reduced ovarian reserve (Tong, Wallace, & Burger 2003).

REFERENCE	Seifer et al. 1997	Corson et al. 1999	Penarrubia et al. 2000	Yong et al. 2003	Kwee et al. 2003	Muttukrishna et al. 2005
POPULATION	ART	ART	ART	ART	ART	ART
STUDY DESIGN	Retrospective	Prospective	Retrospective	Prospective	Prospective RCT	Retrospective
PT/CYCLES	156/178	106	80	58	110	108
CUT-OFF (pg/ml)	45.0	45.0	141.0	N/A (Increment)	N/A (Increment)	≤ 4 oocytes = poor response
OUTCOME MEASURE	RO, PR	PR	RO, PR, CR	RO	RO	RO
CONCLUSION	Low inhibin B (< 45.0 pg/ml) predicts RO and PR	D3 + D10 inhibin B does not correlate with PR	D5 inhibin B predicts CR	b inhibin B of limited value in predicting RO	Inhibin B increment correlates best with RO	Delta inhibin B (in EFORT) predicts RO

TABLE 1.5. 4 Inhibin B as a marker of ovarian reserve.

RO = Retrieved oocytes, PR = Pregnancy rate, CR = Cancellation rate, ART = assisted reproduction

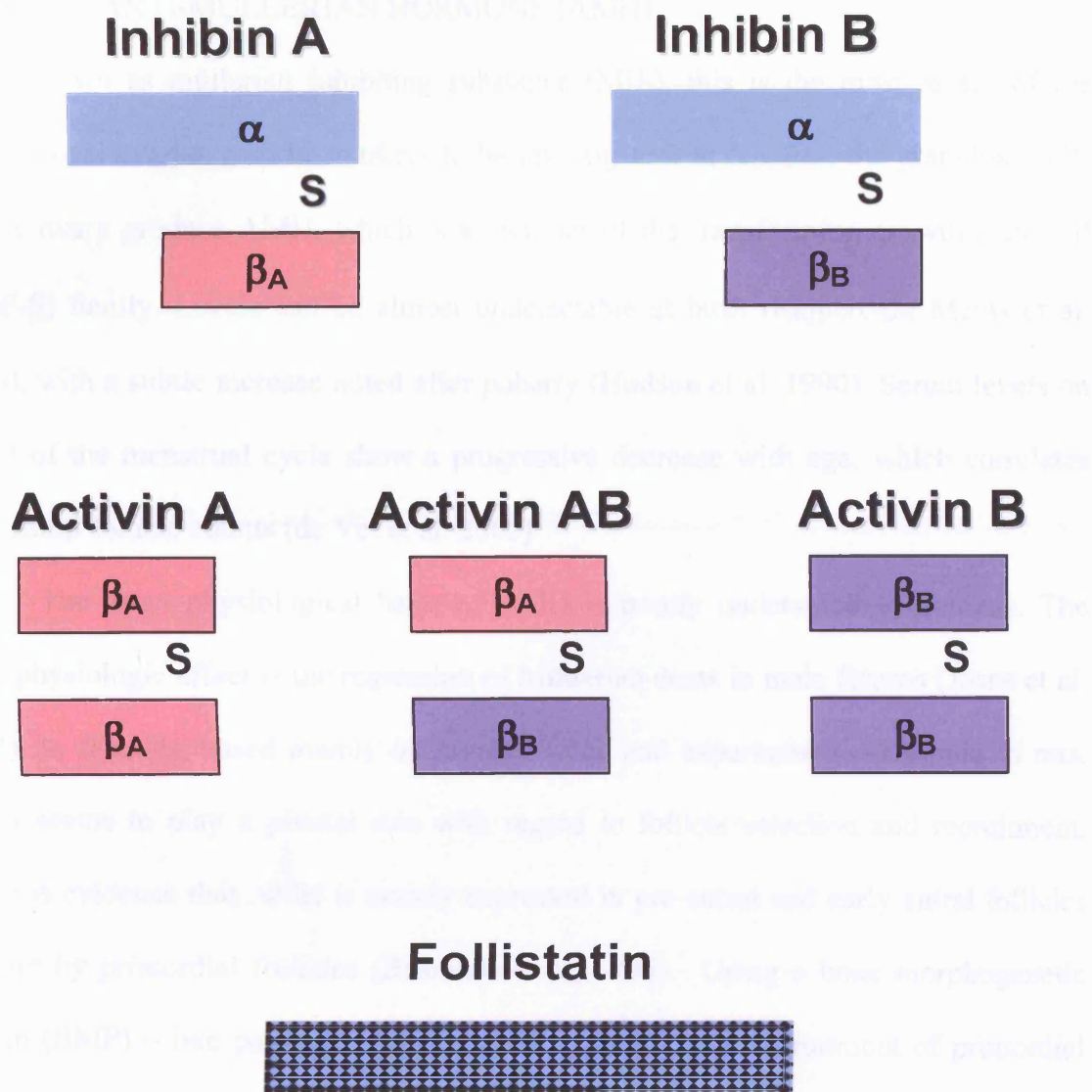


FIGURE 1.5. 3 Structure of inhibin A, inhibin B, activin and follistatin.

Follistatin structure represents a polypeptide.

S = disulphide bridge

1.5.6.4 ANTI-MÜLLERIAN HORMONE (AMH)

Also known as müllerian inhibiting substance (MIS), this is the most recent of the biochemical ovarian reserve markers to be investigated. In females, the granulosa cells of the ovary produce AMH, which is a member of the transforming growth factor - β (TGF- β) family. Levels can be almost undetectable at birth (Rajpert-De Meyts et al. 1999), with a subtle increase noted after puberty (Hudson et al. 1990). Serum levels on day 3 of the menstrual cycle show a progressive decrease with age, which correlates with antral follicle counts (de Vet et al. 2002).

The exact physiological basis of AMH is poorly understood in humans. The main physiologic effect is the regression of Müllerian ducts in male fetuses (Josso et al. 1998). In females, based mainly on in vitro work and experiments performed in rats, AMH seems to play a pivotal role with regard to follicle selection and recruitment. There is evidence that AMH is mainly expressed in pre-antral and early antral follicles and not by primordial follicles (Baarends et al. 1995). Using a bone morphogenetic protein (BMP) – like pathway, AMH signalling inhibits the recruitment of primordial follicles into the pool of growing follicles and also decreases the responsiveness of growing follicles to FSH, thus regulating dominant follicle selection (Durlinger, Visser, & Themmen 2002; Visser & Themmen 2005). Recent progress has been made with regards to elucidating the role of AMH during folliculogenesis in humans. Using immunohistochemistry on ovarian sections obtained from healthy, regularly cycling women, the authors were able to show that AMH expression was similar to the patterns seen in mice (Weenen et al. 2004). AMH also appears to encourage growth of primordial follicles in vitro (Schmidt et al. 2005).

In vivo studies during controlled ovarian hyperstimulation (COH) have shown AMH levels declining gradually, presumably due to the increased number of mature

follicles expressing less AMH (Fanchin et al. 2003), then increase during the mid-luteal phase, reflecting luteal follicle development (Fanchin et al. 2005). These findings suggest that AMH is solely produced by antral follicles capable of growing, and as such serum levels of AMH may represent both the quantity and quality of the ovarian follicle pool (te Velde & Pearson 2002). The potential exists therefore for AMH to provide an accurate estimate of the size of the growing follicular pool – a direct marker of ovarian reserve. Studies have shown an association between reduced baseline serum AMH and poor response to IVF (Seifer et al. 2002; van Rooij et al. 2002). The potential to assess oocyte quality is reflected in studies which show that AMH best predicts clinical pregnancy rate compared to other markers (Hazout et al. 2004) as well as embryo morphology (Silberstein et al. 2006). The relationship between AMH and other markers of ovarian reserve has been assessed, where AMH was found to correlate more strongly with the antral follicle count (AFC) than basal inhibin B, E₂, FSH and LH in an infertile population (Fanchin et al. 2003). Correlation with AFC was also confirmed in patients with PCOS, even into late reproductive age (Piltonen et al. 2005). When compared to these markers in terms of predicting IVF outcome, AMH appeared to be superior (Eldar-Geva et al. 2005; Muttukrishna et al. 2004).

Serum AMH levels have been measured at different times during the menstrual cycle, suggesting minimal fluctuation. (Cook et al. 2000) This implies that AMH levels are relatively convenient to determine, making it an attractive determinant of ovarian reserve (Gruijters et al. 2003). One study in an IVF setting showed improved predictive capacity of day 5 AMH over basal levels (Penarrubia et al. 2005). More recently however, a study has shown that AMH measurement on any day of the menstrual cycle could predict ovarian response in women undergoing IVF (La Marca et al. 2007). Normative data have been published (Cook et al. 2000), and it would appear that AMH expression is relatively stable throughout the menstrual cycle (La Marca et al. 2006). A

recent study has suggested that inter-cycle variability is minimal (Fanchin et al. 2005). The assays are expensive, though sensitive and specific (Long et al. 2000). Newer assays are being developed, which should help address the issues of expense, inter-assay variability and the setting up of standardised reference values (Al Qahtani et al. 2005).

The potential of AMH as a direct marker of ovarian reserve, and its relatively stable expression during the menstrual cycle, would appear to justify further validation. More research is required into the relationship between AMH and ovarian follicle dynamics, and as a marker of ovarian reserve in the general population before allowing its entry into clinical practice.

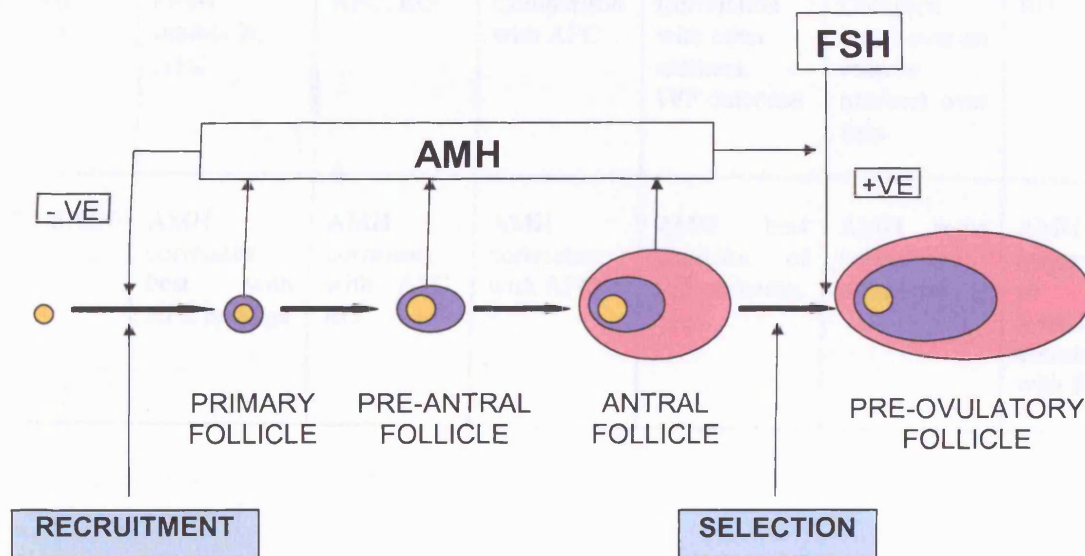


FIGURE 1.5. 4 Role of AMH in folliculogenesis.

Granulosa cells from the developing antral follicles produce AMH, which has an inhibitory effect on follicle recruitment and inhibits the stimulatory effect of FSH on pre-antral and antral follicles, thus influencing dominant follicle selection.

-VE = negative feedback, +VE = positive feedback

REFERENCE	DeVet et al. 2002	Van Rooij et al. 2002	Fanchin et al. 2003	Hazout et al. 2004	Van Rooij et al. 2005	La Marca et al. 2007
POPULATION	General	ART	ART	ART	General with proven fertility	ART
STUDY DESIGN	Longitudinal	Prospective	Prospective	Retrospective	Longitudinal cohort	Prospective
NO/CYCLES	54	119	75	109	81	48
OUTCOME MEASURE	bFSH, inhibin B, AFC	AFC, RO	Comparison with AFC	Correlation with other markers, IVF outcome	Compare basal ovarian reserve markers over time	RO
CONCLUSION	AMH correlates best with AFC and age	AMH correlates with AFC, RO	AMH correlates with AFC	AMH best predictor of IVF outcome	AMH most consistent parameter	AMH (regardless of when measured) correlates with RO

TABLE 1.5. 5 AMH as a marker of ovarian reserve.

RO = retrieved oocytes, α = proportional, AFC = antral follicle count, ART = assisted reproduction

1.5.7 DYNAMIC OVARIAN RESERVE TESTS

1.5.7.1 INTRODUCTION

The recognised limitations of basal biochemical markers of ovarian reserve has led to the development of a number of dynamic ovarian tests in an attempt to “unmask” patients whose diminished ovarian reserve may have been missed using basal markers (Scott, Jr. & Hofmann 1995).

1.5.7.2 GnRH AGONIST STIMULATION TEST (G-test)

This test evaluates the oestradiol serum concentration change from cycle day 2 to day 3 after administration of a GnRH agonist. This is administered at a supraphysiologic dose, causing a temporary increase in pituitary secretion of FSH and LH, to which the ovaries respond by releasing oestradiol (Sharara, Scott, Jr., & Seifer 1998).

Early studies were able to show a limited ability for the early rise in oestradiol concentration to predict ovarian response during IVF, albeit with different cut-off values (Avrech et al. 1996; Padilla 1995; Winslow et al. 1991). Others have looked at the sum of stimulated and basal FSH concentrations with some success, but was not superior to bFSH (Galtier-Dereure et al. 1996). In a significant study, the actual increase in oestradiol concentration from day 2 to day 3 (ΔE_2) following GnRH agonist administration on day 2 was a better predictor of ovarian reserve than age, basal FSH and FSH: LH ratio (Ranieri et al. 1998). The same authors were also able to demonstrate that such an assessment would also allow effective drug regimen selection for IVF (Ranieri et al. 2001).

As inhibin B is considered a direct marker of ovarian reserve, combining it with the G-test might improve the predictive value of the test for IVF outcome. In fact,

changes in the concentrations of inhibin B and oestradiol have both been shown to correlate highly with the ovarian response to stimulation for IVF treatment (Ravhon et al. 2000). A recent study used single or repeated G-test and compared it with other markers of ovarian reserve. Whilst the G-test was able to predict poor response in IVF, it was poor at predicting ongoing pregnancy, especially compared to AFC and inhibin B. Furthermore, there seemed to be no advantage in using repeated tests (Hendriks et al. 2005b).

The G-test appears to have much promise as a dynamic test of ovarian reserve but has not yet been validated outside an assisted reproductive setting. Furthermore the cost of performing the test has limited its acceptability in the general infertility population.

REFERENCE	Padilla et al. 1990	Winslow et al. 1991	Ranieri et al. 1998	Ravhon et al. 2000	Hendriks et al. 2005b
POPULATION	ART	ART	ART	ART	ART
STUDY DESIGN	Comparative	Prospective	Prospective	Prospective	Prospective
PT/CYCLES	100	228	177	37	57
CUT-OFF pmol/l	Day2-day 5 ratio = 2	ΔE_2 100	ΔE_2 180	ΔE_2 100	ΔE_2 200
OUTCOME MEASURE	PR	PR	RO	RO/FSH dose	Ovarian response
CONCLUSION	E_2 response predicts PR	ΔE_2 predicts PR	ΔE_2 predicts RO	ΔE_2 correlates with ovarian response	ΔE_2 predicts poor response but not ongoing pregnancy

TABLE 1.5. 6 G-test as a marker of ovarian reserve.

PR = Ongoing pregnancy rate, RO = retrieved oocytes, ART = assisted reproduction

ΔE_2 = difference between stimulated and baseline oestradiol levels

1.5.7.3 CLOMIPHENE CITRATE CHALLENGE TEST (CCCT)

First described by Navot in 1987, this test involves the administration of 100mg clomiphene citrate on cycle days 5-9, and the determination of FSH (or inhibin) concentrations on days 3 and 10 (Navot, Rosenwaks, & Margalioth 1987). In women with normal ovarian reserve, the overall increase in oestradiol and inhibin production by the developing follicles should be able to overcome the oestrogen antagonist effect of clomiphene on the H-P-O axis, and suppress FSH levels back into the normal range by day 10.

Evidence for the physiologic suppressive effect of inhibin was supported by a study, which showed reduced inhibin B levels following the CCCT in women with reduced ovarian reserve (Hofmann, Danforth, & Seifer 1998). In this way, the CCCT may be more indicative of oocyte quality than quantity. Nevertheless, a quantitative relationship between the CCCT and follicle density has been shown (Gulekli et al. 1999).

Several studies have now been published which show that women with a normal test respond better to ovarian stimulation, while an excessive response predicted a poor outcome to controlled ovarian hyperstimulation for IVF (Csemiczky, Harlin, & Fried 2002; Hofmann et al. 1996; Loumaye et al. 1990; Ng et al. 2005; Tanbo et al. 1992). Correlation with biophysical markers of ovarian reserve (ovarian volume, AFC) has been shown in an infertile population (Erdem et al. 2004). The CCCT also predicts for improved pregnancy rates compared with age alone in an IVF (Scott, Jr. & Hofmann 1995) or general subfertile population (Scott, Jr. et al. 1993).

A retrospective study of women under 40 years of age revealed an inverse relationship between the likelihood of successful pregnancy and CCCT findings, with no definite threshold being identified for FSH beyond which no pregnancy could be achieved (Yanushpolsky et al. 2003). The patient's age should not be ignored however,

as pregnancy rates still diminish in patients with advancing age despite a normal CCCT (Scott et al. 1995). This limitation renders the test, although highly specific, of low sensitivity (26%), albeit higher than that of bFSH (Barnhart & Osherooff 1998).

There has been renewed interest in the evaluation of the CCCT. In a recent meta-analysis, there appeared to be no advantage of the CCCT over bFSH in predicting clinical pregnancy (Jain, Soules, & Collins 2004). Reproducibility has been addressed, as repeating the CCCT predicts poor outcome just as well as a single test, but neither single or repeat CCCT performed better than a combination of other markers (Hendriks et al. 2005a). Another investigator however detected significant intercycle variability with the CCCT, which correlated with reduced ovarian reserve (Kwee et al. 2004).

These results are possibly related to the fact that the exact relationship between elevated day 3 FSH levels and day 10 levels following the CCCT remains to be elucidated (Scott et al. 1995). Until this question is answered from further prospective study, the value of performing the test in the presence of a raised bFSH must remain in doubt.

REFERENCE	Navot et al. 1987	Scott et al. 1995	Hofmann et al. 1996	Yanushpolsky et al. 2003	Hendriks et al. 2005a
POPULATION	ART Age > 35	General infertility	ART	ART Age < 40	ART
STUDY DESIGN	Prospective	Comparative	Retrospective	Retrospective	Prospective
PT/CYCLES	51	588	219	353/483	63
CUT-OFF IU/L	26	10 (day 3 and/or day 10)	16 (day 3 and/or day 10)	10 (day 3 and/or day 10)	Day 10 FSH and inhibin B
OUTCOME MEASURE	PR	PR	PR	DR	Ovarian response
CONCLUSION	CCCT predicts PR	CCCT predicts PR independent of age	CCCT predicts PR	CCCT predicts IVF outcome independent of bFSH	CCCT predicts poor response, but not superior to combination of bFSH and AFC

TABLE 1.5. 7 CCCT as a marker of ovarian reserve.

PR = pregnancy rate, CR = cancellation rate, DR = delivery rate, RO = retrieved oocytes

1.5.7.4 EXOGENOUS FSH OVARIAN RESERVE TEST (EFORT)

This test combines bFSH with the rise in oestradiol (ΔE_2) over a 24-hour period after administration of a standardised dose (300IU) of purified FSH on day 3. More recently, other markers have been incorporated as part of the test (inhibin B, AMH, AFC), which adds to the evaluation of these markers as it applies to follicle dynamics (Hohmann et al. 2005).

Using this test, one group was able to improve the predictive value of bFSH alone for IVF outcome in stimulated cycles (Fanchin et al. 1994). A randomised, prospective study in which basal and dynamic tests of ovarian reserve were compared revealed that EFORT was the best predictor of ovarian reserve (Kwee et al. 2003). More recently, various biochemical and biophysical markers of ovarian reserve were assessed as part of the EFORT. This study revealed that AFC, basal AMH and stimulated inhibin B provided good predictive capacity for oocytes retrieved (Eldar-Geva et al. 2005). In another study, stimulated levels of inhibin B correlated better with age than basal markers (Pastor et al. 2005). Further prospective evaluation is required to validate this test properly.

1.5.7.5 HMG STIMULATION TEST

In comparison to other stimulation tests, the HMG test provides perhaps the greatest ovarian stimulation. In the most recent study using this test, basal values of FSH, E_2 and inhibin were compared with hormonal and ultrasound parameters performed after 5 days stimulation with HMG (Fabregues et al. 2000). Although the predictive value of the rise in E_2 for response to IVF was better than bFSH alone, it was not very specific. Furthermore, the ability to predict pregnancy was less than that provided by the woman's age (Fabregues et al. 2000). These results, and the expense of performing it

may explain the relative lack of popularity in performing this dynamic test in routine practice (Sharif et al. 1998).

REFERENCE	Fanchin et al. 1994 (EFORT)	Fabregues et al. 2000 (HMG)	Kwee et al. 2003 (EFORT)	Eldar-Geva et al. 2005 (EFORT)
POPULATION	ART	ART	ART	ART Age < 38
STUDY DESIGN	Prospective	Prospective	Prospective RCT	Prospective
PT/CYCLES	52	80	110	56
CUT OFF	$\Delta E_2 > 30\text{pg/ml}$; bFSH < 11 IU/L	NS	N/A (Increment)	N/A
OUTCOME MEASURE	Ongoing PR, CR	PR, CR	RO Compared to CCT	RO, pregnancy
CONCLUSION	ΔE_2 superior to bFSH in predicting outcome	ΔE_2 predicts CR but not superior to age in predicting PR	Inhibin B increment in EFORT best predictor of RO	Inhibin B increment predicts RO, but not pregnancy

TABLE 1.5. 8 EFORT and HMG as markers of ovarian reserve.

ART = assisted reproduction, PR= pregnancy rate, CR = cancellation rate, RO = retrieved oocytes,

NS = not specified, N/A = not applicable

1.5.7.6 RESPONSE TO CONTROLLED OVARIAN HYPERSTIMULATION

Regardless of which method is used to predict ovarian response in IVF, one could argue that the final assessment lies in the patient's response to controlled ovarian hyperstimulation (COH). The concept of differentiating a patient's response to gonadotrophin stimulation into high, intermediate or low developed many years ago is still applicable today (Jones, Garcia, & Rosenwaks 1984). Although this group of patients are distinct from the general subfertile population, studies in this area have provided useful insight into the concept of ovarian reserve. One might consider it to be an extended form of dynamic testing.

Of particular interest, distinct cohorts of infertile women, with regular cycles (de Boer et al. 2003) and in addition normal basal FSH levels who do not respond to COH (poor responders), have been shown to develop premature ovarian failure (Farhi et al. 1997; Lawson et al. 2003; Nikolaou et al. 2002). This potential relationship has been challenged by others (De Sutter & Dhont 2003), especially if the poor response is detected at a young age (Hanoch et al. 1998). Poor responders also seem to exhibit characteristics of reduced ovarian reserve when they are tested following a poor response to COH (Beckers et al. 2002). Despite these findings, women with a poor response to COH in the 1st IVF cycle can still have a normal response in subsequent cycles (Klinkert et al. 2004). What adds to the difficulty of interpreting these events is the lack of a uniform definition for poor response among different institutions. It would seem that the most widely used definition pertains to the number of oocytes retrieved in a first or subsequent cycle (Hellberg, Waldenstrom, & Nilsson 2004).

Accurately identifying poor responders to IVF remains the focus of extensive and ongoing research, but effective treatment has proven to be extremely difficult (Lashen et al. 1999; Tarlatzis et al. 2003). Whilst it does seem that repeated stimulation cycles are not detrimental to ovarian reserve (Serna & Garcia-Velasco 2005), there is

recent evidence to support the notion that natural or semi-natural cycle IVF may be just as effective as COH, especially in younger patients (Castelo et al. 2005; Morgia et al. 2004).

Poor response to COH has been attracting considerable interest recently as a marker of ovarian ageing. This is likely to have a considerable impact on the long-term quality of life of patients. Although the model it provides is attractive, in that it involves a dynamic test with strictly controlled conditions and outcomes, it is limited by the fact that application to the general population is not appropriate.

REFERENCE	Farhi et al. 1997	Nikolaou et al. 2002	De Boer et al. 2003
POPULATION	ART	ART	ART
STUDY DESIGN	Retrospective	Controlled retrospective Cohort	Retrospective cohort
PT/CYCLES	12	12	4601
CUT-OFF (pg/ml)	No follicular development > 12mm Δ Peak E_2 < 350 pmol/L	No follicular development > 10mm Peak ΔE_2 < 300 pmol/L	> 3 oocytes retrieved at first attempt
OUTCOME MEASURE	Onset of POF	Onset of POF	Onset of POF
CONCLUSION	Non-response to COH related to POF	Strong association between non-response and POF	Poor response can predict risk of POF

TABLE 1.5. 9 Poor response to Controlled Ovarian Hyperstimulation as a predictor of POF.

POF = Premature ovarian failure, ART = assisted reproduction, ΔE_2 = delta oestradiol

1.5.8 BIOPHYSICAL PARAMETERS

1.5.8.1 INTRODUCTION

These tests offer an opportunity to assess follicle dynamics directly by examining ovarian morphology, and thus are considered direct measures of ovarian reserve. One may deduce that the number of remaining follicles in the ovary is related to fertility or the likelihood of entering menopause (Faddy et al. 1992). The routine presence of transvaginal ultrasound in general fertility and gynaecology clinics may also contribute to the increased popularity of these methods.

1.5.8.2 ANTRAL FOLLICLE COUNT (AFC)

Since the relationship between declining AFC and increasing age was first described (Ruess et al. 1996), many attempts have been made to establish a relationship between AFC and ovarian reserve. The assumption is that the number of antral follicles originating from the cohort of growing follicles also correlates with the number of primordial follicles, or ovarian reserve (Scheffer et al. 1999). AFC is usually defined as the number of follicles less than 10mm in diameter detected by ultrasound in the early follicular phase.

Studies in women with proven fertility reveal a continual decline of AFC with age, which correlates better with chronological age than other hormonal and ultrasound markers (Ng et al. 2003; Scheffer et al. 2003). In this population, it appears that AFC correlates best with other markers of ovarian reserve especially in older age groups (Erdem et al. 2003; Kline et al. 2005).

AFC has been shown to be predictive of ovarian response (Chang et al. 1998a; Chang et al. 1998b; Frattarelli et al. 2003; Hsieh, Chang, & Tsai 2001; Muttukrishna et al. 2005; Nahum et al. 2001; Ng, Tang, & Ho 2000; Pohl et al. 2000; Tomas, Nuoju-

Huttunen, & Martikainen 1997), and can predict pregnancy loss in patients undergoing IVF (Elter et al. 2005). This predictive capacity seems to improve with advancing age (Klinkert et al. 2005). Compared with other markers of ovarian reserve, the AFC may be the best predictor of response to exogenous gonadotrophins (Bancsi et al. 2002) and correlates independently with the number of oocytes retrieved during IVF (Yong et al 2003). A recent meta-analysis was conducted which compared the performance of AFC with bFSH to predict poor ovarian response in IVF. The results showed that AFC was superior at predicting poor ovarian response, but was not very good at predicting non-pregnant outcome (Hendriks et al. 2005c). These poor responders, as predicted by AFC, do not benefit from increasing the starting gonadotrophin dose (Klinkert et al. 2005). Kwee and colleagues conducted a randomised, prospective study that compared antral follicle count (AFC), basal ovarian volume (BOV), the exogenous FSH ovarian reserve test (EFORT) and the clomiphene citrate challenge test (CCCT), with respect to their ability to predict poor and hyper responders. They concluded that AFC performed well as a test for ovarian response, being superior or at least similar to what they described as complex expensive and time consuming endocrine tests, thus supporting the use of AFC in general fertility practice (Kwee et al. 2007).

Reproducibility has been addressed and described as being moderate, with decreasing agreement at higher AFC (Scheffer et al. 2002). However this study was limited to two observers. Intercycle variability has also been described as moderate in a fertile population (Scheffer et al. 1999). Others have shown that intercycle variability was greater than interobserver variations, suggesting a biological variation of AFC from cycle to cycle (Hansen et al. 2003). The only prospective assessment of intercycle variability in a general subfertile population revealed that it increased with younger age (Elter, Sismanoglu, & Durmusoglu 2005). As has been reported by other investigators, identification of an optimal cycle for stimulation did not predict significantly better

outcomes, and as such a single measurement of AFC is recommended (Bancsi et al. 2004; Hansen et al. 2003). There has been recent debate however regarding the optimal time in the cycle to perform AFC. Some argue that in order to detect developing follicles optimally, AFC should be assessed from day 5-7 as opposed to the early follicular phase (Durmusoglu et al. 2004; Kline et al. 2005). As would be expected, there also appears to be a link between AFC and the age of onset of menopause, yet the exact relationship remains to be defined (Broekmans et al. 2004).

Overall the results of these studies show considerable promise for the use of AFC in the estimation of ovarian reserve. The procedure is relatively inexpensive and easy to perform, and can provide information immediately - before biochemical results are obtainable. More data is required in different cohorts and a more rigorous assessment of interobserver variability is required before it becomes part of routine clinical practice.

REFERENCE	Bancsi et al. 2002	Yong et al. 2003	Ng et al. 2003	Klinkert et al. 2005	Hendriks et al. 2005	Kwee et al. 2007
POPULATION	ART	ART	Fertile women aged 17-44	ART Aged 38-46	ART	ART
STUDY DESIGN	Prospective	Prospective	Prospective	Observational	Meta- analysis	Prospective Randomised Comparison
PT/CYCLES	120	58	119	221	11 studies	110
CUT OFF/	2-5	2-10	< 10	< 5mm	Variable	Poor response < 6 follicles
OUTCOME MEASURE	OR, PR	RO	Correlate with age	Predict OR in older age group	Poor response, Non- pregnancy	Number of follicles obtained
CONCLUSION	AFC superior to age and basal endocrine markers	Luteal phase AFC predicts RO, but bFSH superior	AFC best correlates with age	AFC predicts OR better than age and bFSH	AFC superior to FSH in predicting poor OR	AFC superior or at least equal to OV, EFORT or CCCT

TABLE 1.5. 10 Antral follicle count as a predictor of ovarian reserve.

CR = cancellation rate, RO = retrieved oocytes, OR = ovarian response, PR = pregnancy rate, OV = ovarian volume, EFORT = exogenous FSH ovarian reserve test, CCCT = clomiphene citrate challenge test.

1.5.8.3 OVARIAN VOLUME (OV)

The human ovary is a dynamic organ, which changes in size and activity throughout its lifetime. Mean OV increases from 0.7cm³ at age 10 years to 5.8cm³ at age 17 years (Ivarsson, Nilsson, & Persson 1983). Although there is limited data on OV in normal fertile women (Lass & Brinsden 1999), studies suggest that there are no major changes in OV during the reproductive period in individual women until the premenopausal period (Christensen, Boldsen, & Westergaard 1997; Ng et al. 2003). Ovarian size has been shown to decrease in women > 40 years old, a trend which is apparently unaffected by parity (Andolf et al. 1987; Oppermann, Fuchs, & Spritzer 2003). The largest study to address the relationship between age and ovarian volume showed a progressive decline in OV from age 30 to 70, with a dramatic drop in OV occurring after the menopause (Pavlik et al. 2000). These findings have led to the evaluation of OV as a useful marker of menopausal status (Flaws et al. 2000; Flaws et al. 2001), with good correlation being shown between OV, menopausal status and age (Giacobbe et al. 2004).

With regards to assessment of ovarian reserve, an early study found a correlation between OV and reproductive outcome in IVF cycles, but no correlation was found with chronological age (Syrop, Willhoite, & Van Voorhis 1995). Stronger correlations have since been shown between OV and controlled ovarian hyperstimulation (COH) (Lass et al. 1997; Sharara & McClamrock 1999). Small OV (<3cm³) is associated with a poor response to HMG and a very high cancellation rate during IVF (Syrop et al. 1999). One study was able to show a relationship between OV and the number of follicles before stimulation, but not the number of oocytes retrieved (Tomas, Nuojuua-Huttunen, & Martikainen 1997), a finding that has been confirmed elsewhere (Dumesic et al. 2001; Tinkanen et al. 1999). When likelihood ratios are calculated from some of these studies, the overall conclusion is that OV is not a good predictor of pregnancy (Bukman &

Heineman 2001), but when a cut-off value of 3cm³ is used, OV can be predictive of failure of follicular stimulation (Lass & Brinsden 1999).

More recently, studies in an IVF setting have shown that a mean ovarian volume (MOV) of < 2cm³ (Frattarelli et al. 2004) and mean ovarian diameter < 20.6 mm (Vladimirov, Tacheva, & Kalinov 2004) correlates with higher cancellation rates. When compared with other markers of ovarian reserve as part of a CCCT, OV performed well in predicting ovarian response (Erdem et al. 2004).

Wallace and colleagues have recently proposed a model using ovarian volume to predict reproductive age. By estimating the mean primordial follicle population in women aged 25 to 51 years using the Faddy-Gosden equation (Faddy & Gosden 1996), and correlating it with mean ovarian volume at each chronological age as described by Pavlik (Pavlik et al. 2000), reproductive age (age at menopause in this model) can be estimated (Wallace & Kelsey 2004). Others have produced a quadratic model which shows that ovarian volume follows a continuously increasing rate of decline after age 30 (Tufan, Elter, & Durmusoglu 2004). These models make a critical assumption that primordial follicle populations are fixed at birth, a notion that has recently been challenged (Johnson et al. 2004). Correlation has been shown between decreasing MOV and age with inhibin B and FSH in women aged 35-50 (Vural, Vural, & Yucesoy 2005). Whether or not ovarian volume can accurately reflect primordial follicle numbers or indeed follicle quality is questionable. The latter may be more important if reproductive age is to be correlated with fertility outcome.

Intra and inter-observer variations in measurement of OV have been reported by some as being small, ensuring reproducibility (Higgins et al. 1990; Lass et al. 1997). Intercycle variability has recently been reported as being significant in a general infertile population, which appeared to be independent of age (Elter, Sismanoglu, & Durmusoglu 2005).

OV estimation using conventional ultrasound is inexpensive and relatively easy to perform. A recent meta-analysis compared the predictive capacity of OV towards poor response with AFC in an IVF setting. In this study, both parameters were found to have a poor overall accuracy for predicting non-pregnant outcome. However, OV was found to be inferior to AFC, thus leading to the recommendation that AFC and not OV be the first choice in evaluating patients prior to IVF (Hendriks et al. 2007).

Perhaps the main limitation regarding the use of ovarian volume measurements at present in clinical practice is the lack of data regarding OV measurements in the general population, both fertile and infertile, across different age cohorts. When this is achieved, ovarian volume measurements are likely to play a more important role in the clinical estimation of ovarian reserve.

REFERENCE	Lass et al. 1997	Sharara and McClamrock 1999	Pavlik et al. 2000	Flaws et al. 2001	Frattarelli et al. 2004	Hendriks et al. 2007
POPULATION	ART	ART	General	General	ART	ART
STUDY DESIGN	Prospective	Prospective	Prospective cohort	Cross-sectional	Prospective cohort	Meta-analysis
PT/CYCLES	140	109	13,963	50	267	1146
CUT OFF/ CM ³	MOV 3cm ³	MOV 3cm ³	N/A	Variable	MOV < 2	Variable
OUTCOME MEASURE	PR, CR	CR	Age – related changes in OV	Menopausal status	RO	Poor ovarian response, non-pregnancy
CONCLUSION	Low MOV correlates with CR	Low MOV correlates with CR	OV progressively decreases from age 30	OV has similar sensitivity/specificity to bFSH and age	OV correlates with RO	AFC superior to OV in prediction of poor response

TABLE 1.5. 11 Ovarian volume as a predictor of ovarian reserve.

MOV = Mean ovarian volume, PR = pregnancy rate, CR = cancellation rate,

RO = retrieved oocytes

1.5.8.4 OVARIAN STROMAL BLOOD FLOW (OSBF)

The rationale for this test lies in the supposition that the primordial follicles in the ovary have no independent capillary network, and therefore depend on their proximity to the stromal vessels for their supply of nutrients and hormones (Findlay 1986). Studies have shown a relationship between peri-follicular blood flow doppler indices and oocyte developmental competence/implantation potential (Chui et al. 1997; Huey et al. 1999; Nargund et al. 1996).

A relationship has been established between ovarian stromal blood flow velocity and ovarian follicular response (Zaidi et al. 1996). This was confirmed in a cohort of patients with normal basal FSH undergoing IVF treatment following pituitary suppression, in which mean ovarian stromal peak systolic velocity (PSV) was a better predictor of ovarian responsiveness than age (Engmann et al. 1999), while resistance index (RI) was more predictive in a similar study (Bassil et al. 1997). By assigning a quantitative “total doppler score” based on the number and area of doppler signals in the ovary, some investigators have been able to predict ovarian response in IVF (Popovic-Todorovic et al. 2003). In another study based on a fertile population however, age was not related to mean PSV, although power doppler was not used (Ng et al. 2003). When 3D power doppler was employed, the results implied that OSBF indices declined slowly with age, becoming significantly reduced only in women > 41 years (Ng et al. 2004). The same authors have studied 2D power doppler in an IVF setting and were unable to determine any predictive capacity for OSBF indices with regard to ovarian response (Ng et al. 2005).

A limitation of this technique is that it is very operator dependent. It requires an ultrasonographer of considerable experience to obtain accurately the angle between the ultrasound beam and the intra-ovarian vessels in order to measure peak systolic velocity. This may result in a prolonged scanning time for acquisition of data. It has been

suggested that 3D USS can circumvent this difficulty (Kupesic & Kurjak 2002). Using vascularisation-flow indices, 3D power Doppler USS has been used to show that flow intensity in the ovarian stroma decreases with age in general (Pan et al. 2002) and is less in infertile patients (Kupesic et al. 2003). Another study was unable to show a correlation between these indices and ovarian response during IVF, with inter and intra-observer variability being described as acceptable (Jarvela et al. 2003). More recent applications using 3D power-doppler are now being reported, including measurement of endometrial and subendometrial blood flow, the results of which are very preliminary (Ng et al. 2006).

Further study is required to assess the predictive value and reproducibility of ovarian stromal blood flow measurements compared to other biophysical markers of ovarian reserve before entering routine clinical practice.

REFERENCE	Engmann et al. 1999	Ng et al. 2003	Ng et al. 2005
POPULATION	ART	General Fertile	ART
STUDY DESIGN	Prospective observational	Prospective cohort	Prospective cohort
PT/CYCLES	88	119	136
PARAMETER	PSV	PSV Grouped by age	Mean PSV, RI, PI
MODALITY	2D COLOUR	2D COLOUR	2D POWER
OUTCOME MEASURE	RO, PR	Correlation with age	OR
CONCLUSION	PSV can predict ovarian response	No correlation with age	OSBF indices do not predict OR

TABLE 1.5. 12 Ovarian stromal blood flow parameters.

RO = retrieved oocytes, PR = pregnancy rate, OR = ovarian response PSV = peak systolic velocity, RI = resistance index, PI = pulsatility index

1.5.8.5 THREE DIMENSIONAL ULTRASOUND (3D USS)

It has been suggested that 3D USS can improve the prediction of ovarian response to gonadotrophin stimulation, compared to conventional transvaginal ultrasound with power Doppler imaging. 3D imaging yields a more accurate estimate of OV, endometrial volume, AFC and assessment of ovarian stromal perfusion by 3D power doppler (Kupesic 2001). Initial attempts to evaluate the use of 3D USS in studying low responders to stimulation for IVF proved that the technique was useful in differentiating poor responders who had normal baseline FSH levels (Pellicer et al. 1998). This study did not attempt to compare 3D USS with conventional transvaginal (TV) ultrasound imaging. The role of 3D USS in assessing OSBF has already been addressed in the previous section. To add to this, one study evaluated the use of AFC, OV, stromal area and OSBF as assessed by 3D USS in predicting ovarian response and IVF outcome. In this study, OSBF and AFC seemed to be better predictors of ovarian response than ovarian volume and total stromal area (Kupesic & Kurjak 2002). Reproducibility has been assessed with regard to AFC in a comparison between 2D and 3D USS, and has been described as being adequate (Scheffer et al. 2002).

One significant advantage of 3DUSS over conventional TV USS that has emerged from these studies is the reduction in examination time required for these patients. Imaging data can be stored allowing further assessment to be performed without the presence of the patient. A significant disadvantage is the high cost of 3D USS over conventional USS. More studies are required to compare the predictive ability of both modalities prospectively. Until this data becomes available, 3D USS would not be expected to replace conventional USS for some time to come.

REFERENCE	Pellicer et al. 1998	Kupesic et al. 2002	Kupesic et al. 2003
POPULATION	ART	ART	ART
STUDY DESIGN	Prospective Case-control	Prospective	Prospective
PARAMETERS MEASURED	AFC and OV	AFC, OV, total stromal area, OSBF	FLOW INDEX AFC OV
PT/CYCLE NUMBER	18	56	56
OUTCOME MEASURE	Comparison between normal and low responders	RO, PR	RO, FR, PR
CONCLUSION	AFC but not OV discriminates normal from low responders	AFC and OSBF predicts outcome	Good correlation with age and ovarian response

TABLE 1.5. 13 3D USS as a predictor of ovarian reserve.

RO = retrieved oocytes, PR = pregnancy rate, FR = fertilisation rate,

ART = assisted reproduction

1.5.8.6 OVARIAN FOLLICLE DENSITY (OFD)

The histopathological examination of ovarian biopsy specimens has been advocated as a direct marker of ovarian reserve. Assessment of follicular density from ovarian biopsies taken from 60 subfertile women with normal basal FSH levels showed a decline with increasing age, but the study did not follow up for pregnancy outcome (Lass et al. 1997). A study performed on a cohort of patients with Turner's Syndrome who were undergoing ovarian tissue cryopreservation showed that OFD correlated with levels of FSH (Hreinsson et al. 2002). In a unique study, women over 35 years of age undergoing oophorectomy for uterine pathology had indirect markers of ovarian reserve assessed in a previous cycle (Gulekli et al. 1999). Although a positive correlation was identified between basal serum oestradiol concentrations and follicle density in ovarian tissue, no significant correlation was seen between basal and clomiphene-stimulated FSH levels and OFD (Gulekli et al. 1999). Recent studies have suggested that while OFD may not add additional information in patients with diminished ovarian reserve, the potential exists for it to be more informative in patients with POF (Massin et al. 2004; Vital-Reyes et al. 2006).

A major problem with this technique lies in the possibility that the follicle density measured in a particular specimen (biopsy site or histological section) may not reflect the picture in the rest of the ovary (Lass 2001). This was shown recently, where the follicle density of ovarian biopsy specimens obtained at the time of ovarian tissue cryopreservation varied by more than two orders of magnitude, although there was a significant inverse linear correlation with age (Schmidt et al. 2003). Lambalk and colleagues attempted to investigate this further by performing multiple biopsies of 2 and 5mm and whole ovaries from 5 patients of reproductive age, during operations not involving ovarian pathology (Lambalk et al. 2004). The authors attempted to estimate total ovary follicle number by transforming biopsy counts based on surface area.

Despite this, predictive values based on the biopsies were widely varied (Lambalk et al. 2004). At present, there are no standardised measures for OFD amongst histopathology laboratories. This “correction factor” is necessary to account for that proportion of the ovary not included in the sampling analysis, and may account for the large discrepancy in follicle numbers reported by various laboratories (Tilly 2003).

Another major pertains to acquisition of an adequate specimen, which includes the invasiveness of performing a biopsy and the risk of adhesion formation (Sharara & Scott 2004). One cannot put a patient through a surgical procedure to obtain a biopsy of ovarian tissue of unproven value.

In the specific situation of cancer patients undergoing ovarian tissue freezing for fertility preservation, OFD may be a useful research tool (Poirot et al. 2002). This possibility, as well as progress in estimating the distribution of follicles in the ovarian cortex, still provides some hope for the clinical application of this test in the future (Lass 2004; Sharara & Scott 2004).

1.5.9 SUMMARY OF OVARIAN RESERVE TESTS

ORT has been developed originally in an assisted reproduction setting in the search for more reliable predictors of IVF outcome. FSH is the longest established ORT, and appears to be superior to clinical predictors. The recognised limitations of basal markers such as FSH have led to the evaluation of several other tests of ovarian reserve. Dynamic testing has the potential to unmask poor responders, in that it may reveal patients who would not have a good response to IVF stimulation despite a normal baseline profile. Newer biochemical parameters such as AMH and inhibin B have a sound physiologic basis as direct markers, but are expensive to perform. Biophysical markers such as AFC and ovarian volume are relatively convenient and easy to perform and provide “real time” information on ovarian reserve. However, they require skilled personnel to perform transvaginal ultrasound especially if OSBF is being measured. The expense is increased when 3D USS is considered.

Despite all the options available however, the ideal ovarian reserve test has yet to be identified. A recent systematic review showed that the ORT's known to date have only modest to poor predictive properties with regards to IVF outcome, and as such should not be considered suitable for relevant clinical use (Broekmans et al. 2006). The poor predictive capacity of ORT in the assisted reproduction setting has led to some considerable debate as to the value of routine ovarian reserve testing (Balmaceda & Schwarze 2007; Maheshwari, Fowler, & Bhattacharya 2006). It would be fair to say that the tests perform fairly well as far as predicting response to ovarian stimulation, but can't predict non-pregnant outcome because there are too many variables.

More information is required, using adequately controlled and prospective study designs of sufficient statistical power, outside a fertility setting.

2. AIMS, HYPOTHESES AND STUDY DESIGN

2.1 IN VIVO STUDY

2.1.1 INTRODUCTION

In the previous chapter, several important points were established. These may be summarised as follows:

- There is a rising incidence of breast cancer and a significant proportion of women being treated for breast cancer are of reproductive age at the time of diagnosis.
- These patients represent a distinct cohort with specific concerns regarding their fertility and the possibility of having premature ovarian failure (POF).
- Ovarian reserve testing has become established in the fertility setting, where it is used as a counselling tool and a guide to treatment.
- The ideal ovarian reserve test (ORT) has yet to be identified.
- Very little is known about chemotherapy-induced alterations in ovarian reserve.
- There is a lack of pertinent data relating to ovarian reserve testing in cancer, and information pertaining to ORT in a subfertile population cannot be extrapolated to include patients with cancer, as the pathophysiology is distinct.
- At the moment it is impossible to predict the exact life span of the chemotherapeutically damaged ovary and the reproductive potential of these patients.
- Patients with cancer would benefit from knowledge of their functional ovarian reserve. With this information, ovarian reserve testing could be applied clinically in patients with cancer as a guide to counselling and treatment with regards to reproductive health and fertility preservation.

2.1.2 STUDY AIM AND HYPOTHESIS

The aim of this study was to evaluate ovarian reserve in young women treated by multi-agent cytotoxic chemotherapy for breast cancer by using clinical, biochemical and biophysical parameters. This was based on the hypothesis that cytotoxic drugs used to treat premenopausal patients with breast cancer can cause ovarian damage resulting in diminished ovarian reserve.

2.1.3 STUDY DESIGN

2.1.3.1 INTRODUCTION

Information from previous studies on ovarian reserve in cancer are limited by a heterogeneous case mix (including cancer diagnosis, chemotherapy regimens and age), as well as a lack of prospective, controlled data. For this study, a breast cancer cohort was chosen for several reasons. First, it is the most common cancer in women of reproductive age in the United Kingdom (and therefore relevant) and allows the study to be disease standardised. Second, the chemotherapy regimens used are similar (mainly E-CMF or FEC) and are cyclophosphamide based (thus reducing confounding due to type of chemotherapy used). A control group was chosen that was not only age-matched, but had proven fertility, thus increasing the likelihood that the data obtained on ovarian reserve accurately reflected the normal range in a general fertile population. Finally the methodology employed for ovarian reserve testing was particularly robust in order to ensure reproducibility. A mixed longitudinal and cross sectional prospective case controlled study was designed, which for statistical purposes would need to comprise at least 20 patients and a similar number of controls. Ethical approval was approved for the study to be undertaken comprising 80-100 chemotherapy treated cancer patients and 60-80 age-matched, healthy control subjects.

2.1.3.2 SAMPLE SIZE

Statistical advice was obtained from Dr Rumana Omar (Medical Statistics Department of the Research and Development Directorate, University College London). Based on her recommendations, it was estimated that a sample size of 18 in each group (patients and controls) would have provided a 90% power of detecting a statistically significant difference between the two groups. However to allow for dropouts or missing values the aim was to recruit at least 20 patients and 20 controls.

This recommendation was based on published data regarding ovarian reserve in a general population, as well as in a fertility setting [FSH/LH (Van Montfrans et al. 2000), Oestradiol (Ranieri et al. 1998), Inhibin B (Seifer et al. 1997), MOV and AFC (Chang et al. 1998; Lass et al. 1997)]. There were insufficient data on all other indices of ovarian reserve used in this study in the general population.

PARAMETER	MEAN	S.D.	ABNORMAL CUT-OFF
Basal FSH	6.4 mIU/L	1.5	> 10.0
Basal LH	4.8 mIU/L	1.9	> 22.0
Basal E ₂	180 pmol/L	50.0	< 120.0
ΔE_2	250.2 pmol/L	13.4	< 180.0
Basal Inhibin B	143.73 pg/ml	46.0	< 45.0
Mean Ovarian Volume	6.3 ml	3.1	< 2.9
AFC	11.6	6.2	< 4.0

TABLE 2. 1 Reference ranges used to perform the power calculation.

S.D. = standard deviation.

2.1.3.3 INCLUSION AND EXCLUSION CRITERIA

Inclusion criteria for the study were as follows:

- Any patients with primary breast cancer prior to or after completion of chemotherapy
- Age range 20 to 45 years
- Controls comprised women of the same age group and with at least one childbirth in the past

Exclusion criteria for the study were as follows:

- Patients with recurrent breast cancer
- Patients who had received
 - Zoladex TM within the last 12 weeks
 - Tamoxifen within last 12 weeks
- Controls who had been on the oral contraceptive pill (OCP) within the last 8 weeks
- Patients or controls with
 - Premature ovarian failure
 - Suspected or proven pregnancy
 - Previous hysterectomy
 - Endocrinological illness
 - Poor medical condition

2.1.3.4 STUDY PROTOCOL

In this study, pre-menopausal recipients of chemotherapy for breast cancer were analysed in two groups – a longitudinal arm (group 1) and a cross-sectional arm (group 2). As all patients were recruited from a single centre, the chemotherapy regimens employed were standardised. There were two predominant regimens, both cyclophosphamide-based. The first was E-CMF, which consisted of 4 cycles of epirubicin (E), followed by 4 cycles of cyclophosphamide(C), methotrexate(M) and 5-fluorouracil(F). The second was FEC, which consisted of 6 cycles of 5-fluorouracil, epirubicin and cyclophosphamide. Some patients received taxanes (docetaxel or paclitaxel), usually as part of a therapeutic trial (TANGO).

In group 1, patients were offered ovarian reserve testing prior to receiving chemotherapy. The same patients were then followed up longitudinally by performing further testing immediately following chemotherapy. In group 2, patients were tested for ovarian reserve prior to chemotherapy (some patients were also part of the longitudinal study) and after chemotherapy (once regular menstrual cycles had resumed). Patients with oestrogen-sensitive cancer were recruited provided they had not taken tamoxifen or GnRH analogues (goserelin or leuprolin) for at least the preceding 12 weeks.

Controls were age-matched, had no medical illness, had proven fertility (defined by virtue of having at least one childbirth previously) and had a normal menstrual history. Those on oral contraception were asked to discontinue it and use alternative (barrier) contraception for at least 8 weeks prior to testing.

Informed consent was obtained from all participants. This study received ethical approval from the Joint UCL/UCLH Ethics Committee.

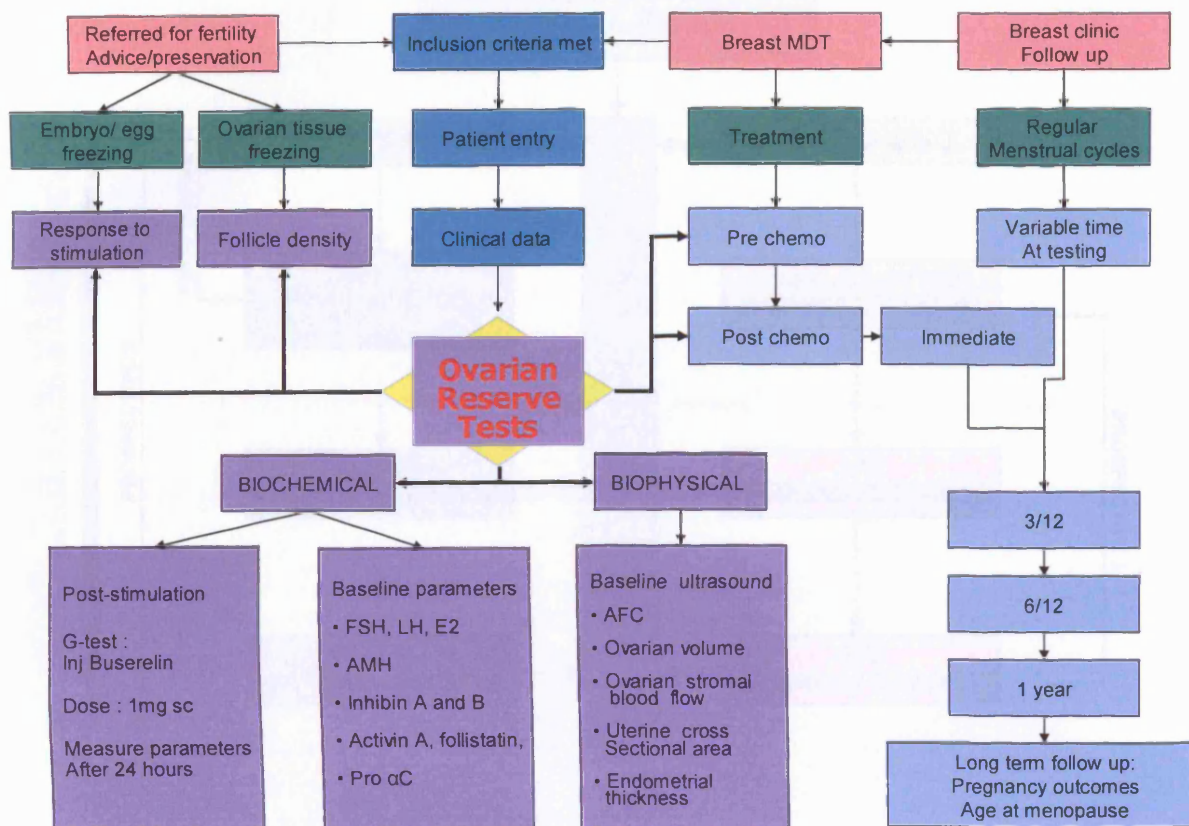


FIGURE 2.1. 1 IN VIVO STUDY DESIGN – PART 1

This diagram illustrates the three main sources of recruitment for the in vivo study. Once the inclusion criteria were met, appropriate consent was obtained and clinical details were recorded. Patients then underwent biochemical and biophysical ovarian reserve tests at various times as shown. Biochemical ovarian reserve tests were performed both as a baseline and as a component of the G-test (see chapter 4- methods) and included FSH, LH, E2, AMH, Inhibins A and B, Activin A, Pro α C and Follistatin. Biophysical ovarian reserve tests included antral follicle count and ovarian volume (total and mean), doppler flow indices (MPI and MPS) and uterine dimensions (CSA and ET). Response to ovarian stimulation and follicle density were not included in this study, but are areas of potential future interest.

COLOUR CODE:

- STUDY ENTRY POINT
- OVARIAN RESERVE TESTS
- TIME OF TESTING

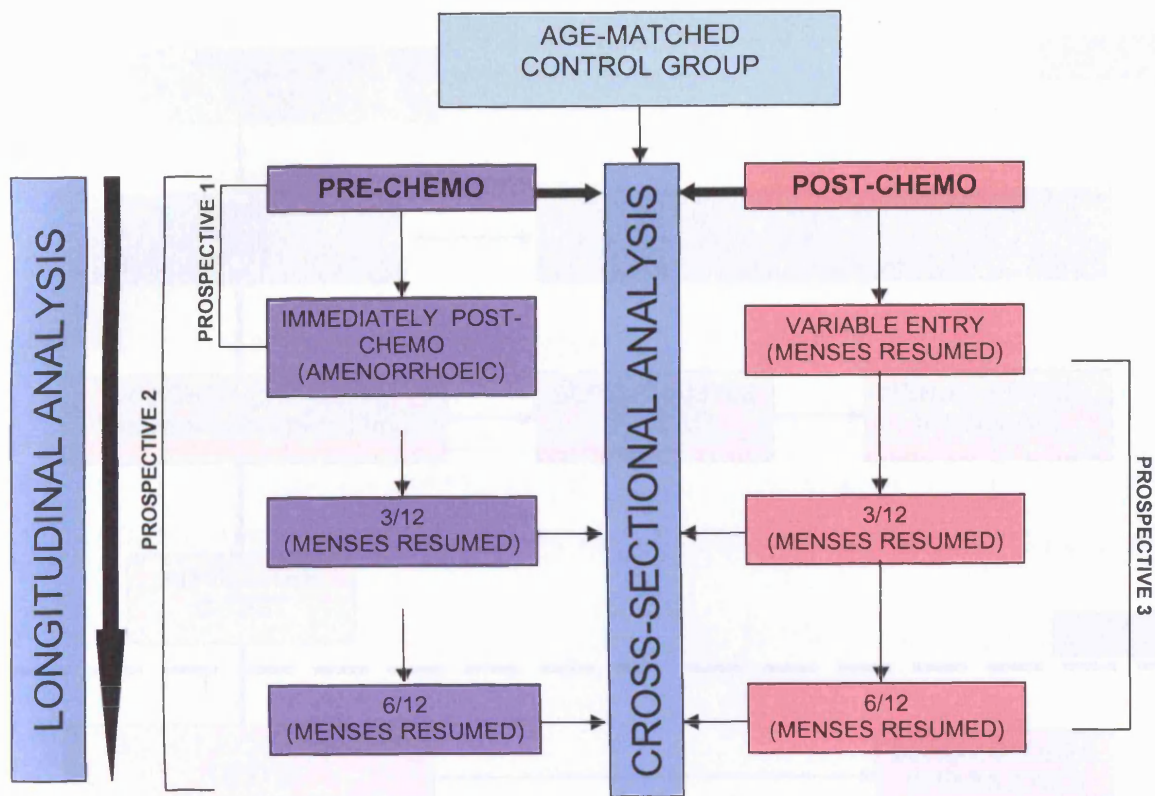


FIGURE 2.1.2 IN VIVO DESIGN - PART 2

This diagram illustrates where patients were grouped in the study. Patient's recruited pre-chemotherapy also contributed to the cross-sectional analysis as shown. There were also three prospective time points studied as shown.

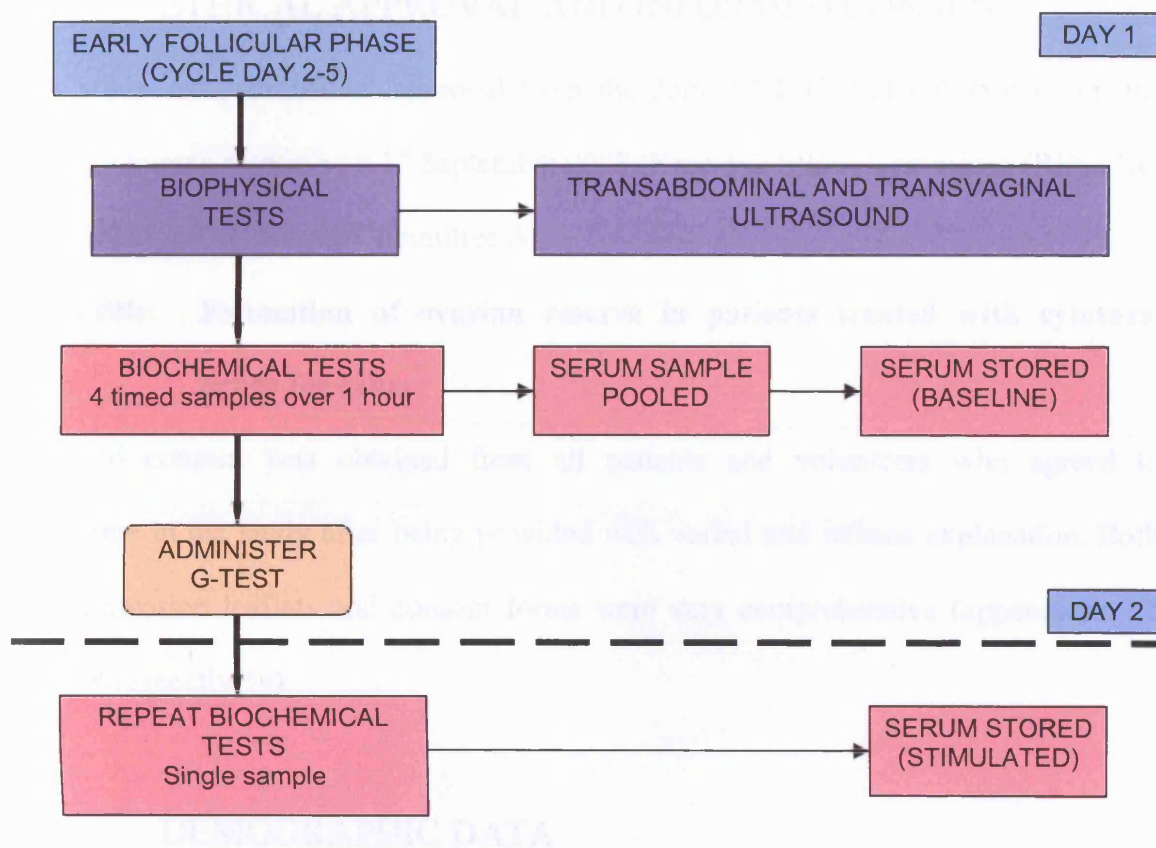


FIGURE 2.1.3 Schedule for ovarian reserve tests

2.1.4 ETHICAL APPROVAL AND INFORMED CONSENT

This study received ethical approval from the Joint UCL/UCLH committees on the ethics of human research on 1st September 2003 (Research Ethics Committee (REC) Ref No: 03/0156; REC Name: Committee A).

Study title: Estimation of ovarian reserve in patients treated with cytotoxic drugs for cancer

Informed consent was obtained from all patients and volunteers who agreed to participate in the study after being provided with verbal and written explanation. Both the information leaflets and consent forms were very comprehensive (appendices 9.3 and 9.4 respectively).

2.1.5 DEMOGRAPHIC DATA

Clinical details of all patients and controls were recorded. This included data such as age, parity, medical and gynaecologic histories, family history of breast and other cancers, premature menopause, social and personal history (including smoking), medications and clinical characteristics such as height, weight and body mass index (BMI).

For patients who had already completed chemotherapy, data included full diagnosis (e.g. stage/grade etc), cancer treatment history and planned treatment and length of remission. For patients who opted to have fertility treatment, data included number of embryos (or eggs) stored, pregnancy and implantation rates, etc. For patients who opted to have ovarian tissue storage, data from the routinely taken histology (biopsy) sample were used, including the number of eggs contained in, or the follicle density of the sample.

2.1.6 DATA STORAGE

The research team was primarily responsible for security and access to data. All data was pseudoanonymised or linked in accordance with the data protection act 1998. Data safety, confidentiality and security were maintained by irretrievably removing names and identifiers. All samples and data were coded with the key available only to the main investigators.

2.2 IN VITRO STUDY

2.2.1 INTRODUCTION

In the previous section, an *in vivo* study was described which was designed to examine the effect of cytotoxic agents on ovarian reserve in patients receiving chemotherapy for breast cancer. As discussed earlier, the mechanism of chemotherapy-induced ovarian failure is poorly understood. One of the parameters involved in this process is the dose of chemotherapeutic agent administered. This is difficult to ascertain accurately *in vivo*, as other variables such as patient age and pre-morbid ovarian reserve must be taken into account. It seemed logical therefore that after performing the *in vivo* measurement, one should examine the effect of these agents on specific ovarian cells and to compare the potency of the drugs used between breast and ovarian cells to investigate the difference. An attempt was therefore made *in vitro* to compare the cytotoxicity of drugs commonly employed in breast cancer on granulosa cells, as the function of these cells could be considered analogous to markers of ovarian reserve.

2.2.2 BACKGROUND

Granulosa cells proliferate with follicular development, and their functional capacity can be estimated in follicular fluid by measuring inhibin and activin (Lau et al. 1999). The secretion of inhibin and activin has been shown to be controlled differentially by gonadotrophins *in vitro* (Muttukrishna, Groome, & Ledger 1997). Steroidogenesis can also be assessed by measurement of oestradiol and progesterone, which has been shown to vary with chronological age from cultured human ovarian granulosa cells (Denkova et al. 2000).

Few studies exist that evaluate the effect of cytotoxic agents used to treat breast cancer on granulosa cell function. One study showed that cyclophosphamide

metabolites, at concentrations achievable in vivo during cyclophosphamide therapy, decrease rat and human granulosa cell function in vitro as assessed by progesterone accumulation (Ataya, Pydyn, & Ramahi-Ataya 1990).

Apoptosis, or programmed cell-death, has been shown to occur in granulosa cells as part of the process of follicular atresia where it is an important determinant of reproductive ageing (Piquette et al. 1994; Tilly et al. 1991; Tilly 1996; Yuan & Giudice 1997). Studies in a reproductive medicine setting have revealed that an increase in the number of luteinised granulosa cells undergoing apoptosis is associated with increased FSH (Seifer et al. 1996) and reduced ongoing pregnancy rates after IVF (Oosterhuis et al. 1998).

Experiments using a rat model have shown that apoptosis-associated signalling pathways are required for chemotherapy-mediated female germ cell destruction (Perez et al. 1997). Furthermore, studies performed on the breast cell line MCF-7 have shown that chemotherapy-induced apoptosis plays a critical role in breast cancer therapy (Simstein et al. 2003).

The cytotoxic effect of chemotherapeutic agents used to treat breast cancer on granulosa cells function can therefore be assessed by measuring hormones and cell integrity assessed by apoptosis.

2.2.3 STUDY AIM AND HYPOTHESIS

The aim of this study was to examine the dose-related effect of cytotoxic agents used to treat breast cancer on granulosa cells, by assessment of granulosa cell function and integrity in vitro. This was based on the hypothesis that cytotoxic drugs used to treat premenopausal patients with breast cancer can cause granulosa cell injury and death.

2.2.4 STUDY DESIGN

2.2.4.1 INTRODUCTION

In order to study the effect of cytotoxic agents on granulosa cells, a breast cell line (MCF-7) was used as a control to establish the dose range under which the LD50 (dose required to kill 50% of the cells) in granulosa cells would lie. Four cytotoxic agents were chosen that represent commonly used drugs in patients with breast cancer. These agents have all been shown to induce apoptosis in the human breast cell line MCF – 7: Doxorubicin (D) and Melphalan (M) (Kugawa et al. 2004), Paclitaxel (P) (Saunders et al. 1997) and Cisplatin (C) (Blanc et al. 2000).

2.2.4.2 STUDY PROTOCOL

The first part of this study involved establishing the dose range for each drug using the MCF-7 cell line. Once the dose range was established, a primary granulosa cell culture was established by isolating these cells from human follicular fluid obtained from patients undergoing in-vitro fertilisation (IVF). Ethical approval for this aspect of the study was obtained from the joint UCL/UCLH ethics committee.

Once both culture conditions were optimised, experiments were performed which involved application of similar dosages of cytotoxic drugs in parallel. After a defined exposure time, the cells were isolated from the culture medium and analysed by flow cytometry. In addition, determining the concentrations of inhibin A and oestradiol released respectively functionally assessed the granulosa cells in culture in the presence of these agents.

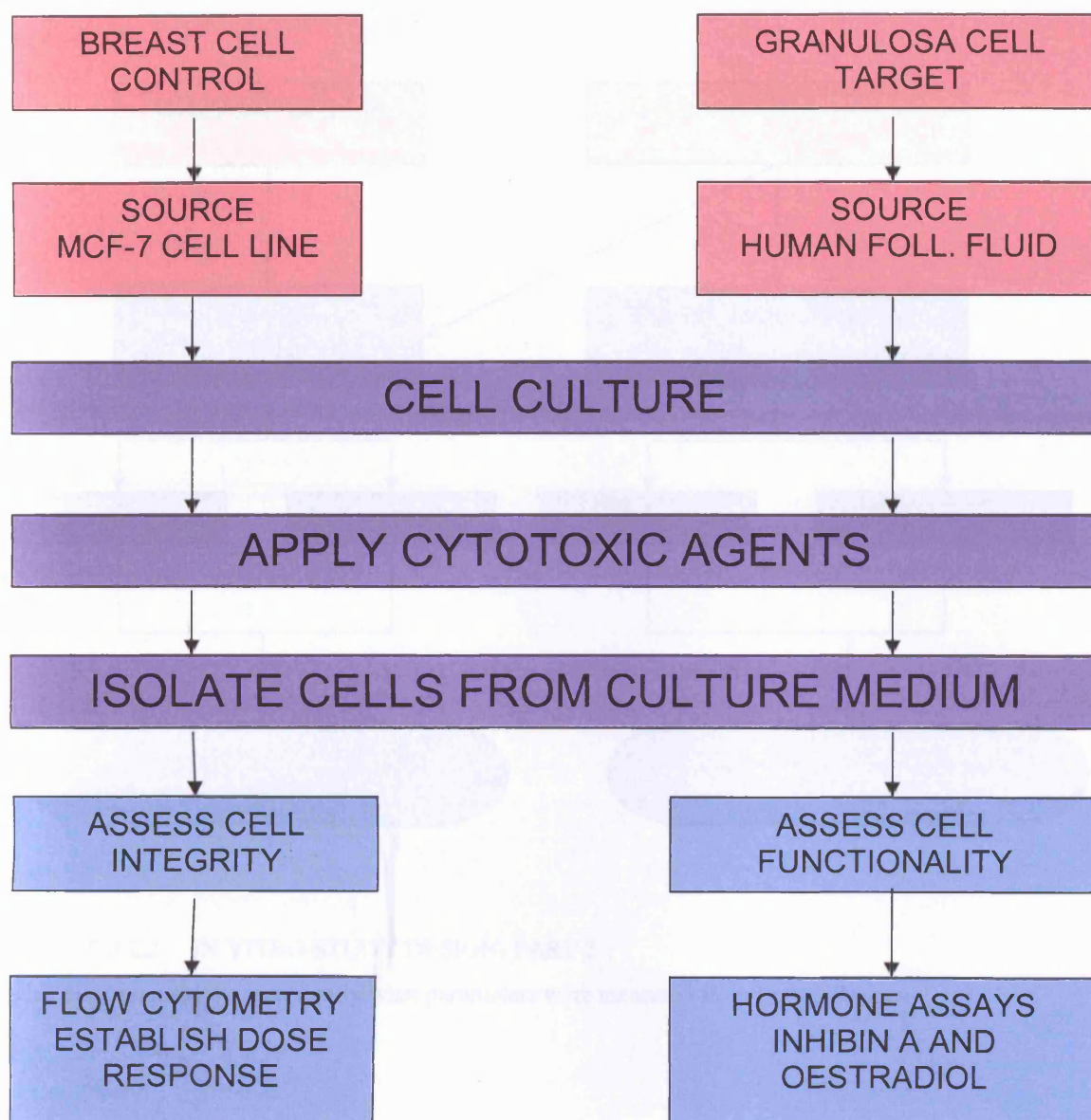


FIGURE 2.2. 1 IN VITRO STUDY DESIGN – PART 1

Cell culture conditions were optimised for both the breast cell line and the granulosa cells prior to simultaneous application of equivalent dosages of cytotoxic agents. Cells were then extracted and cytometric data obtained for both cell types. From this data, dose response curves were obtained. Further information was obtained from the granulosa cells where hormone assays were performed for inhibin A and oestradiol contained in the culture medium.

HUMAN FOLL. FLUID = Human follicular fluid

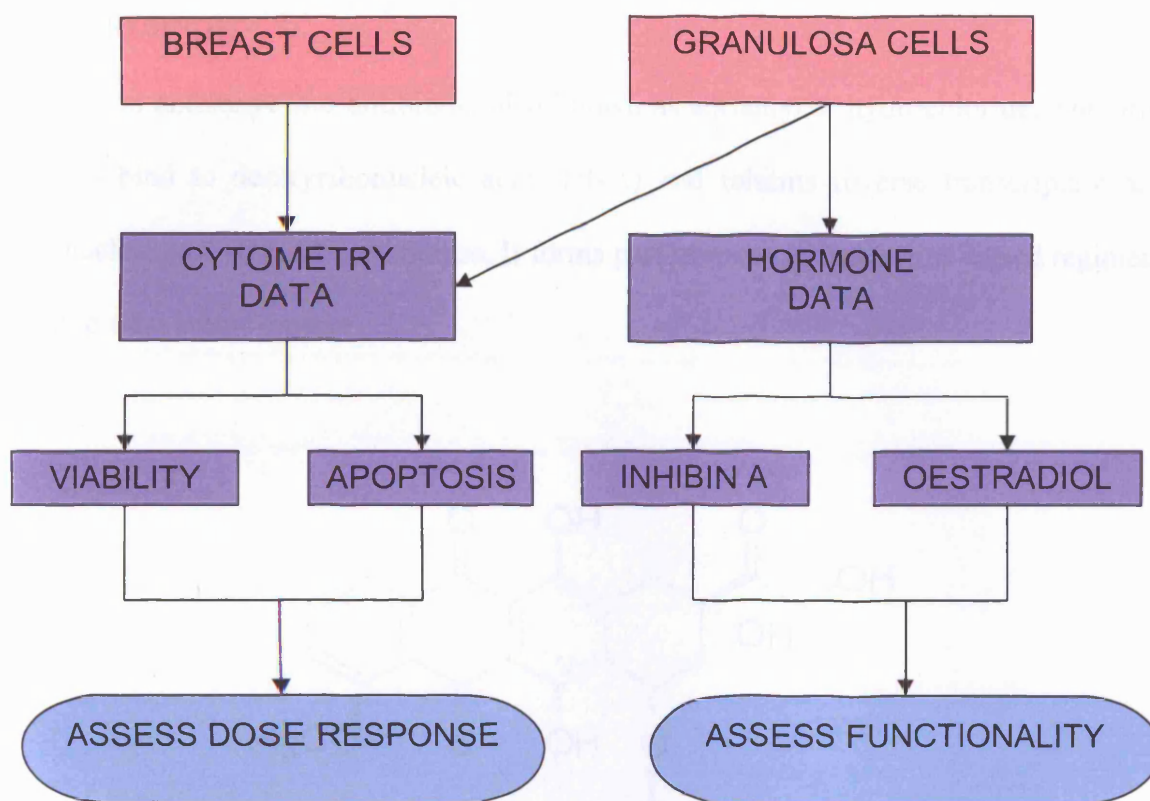


FIGURE 2.2.2 IN VITRO STUDY DESIGN- PART 2

This diagram displays specifically what parameters were measured from both cell types.

2.2.4.3 CYTOTOXIC AGENTS

DOXORUBICIN

This is an anthracycline antibiotic, also known as adriamycin hydrochloride. The drug acts to bind to deoxyribonucleic acid (DNA) and inhibits reverse transcriptase and ribonucleic acid (RNA) polymerase. It forms part of most anthracycline-based regimens used to treat breast cancer.

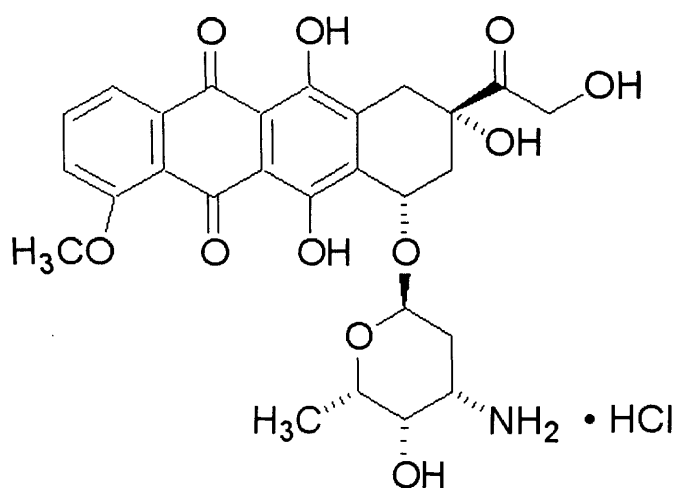


FIGURE 2.2. 3 Structure of doxorubicin.

Molecular Weight (MW) 579.

MELPHALAN

This is a nitrogen mustard derivative that forms DNA intrastrand crosslinks by alkylation of the 5'-(GGC) sequence. It was chosen as the as an alternative to cyclophosphamide in this study. Cyclophosphamide is also a nitrogen mustard derivative, which requires activation by the hepatic cytochrome P-450 enzyme system to become biologically active. For in vitro study, the activated form of the drug – 4-hydroxycyclophosphamide would have to be used. Administration of the activated form has been shown to exert a dose-dependent reduction in progesterone levels in rat

granulosa cells (Ramahi-Ataya et al. 1988). This “activated” form of the drug was very difficult to obtain commercially. Melphalan is considered to be similar to cyclophosphamide in potency and mechanism of action, and has been shown to have activity against breast cancer in vivo (Carpenter, Jr. & Maddox 1983) as well as in vitro against an MCF-7 cell line (Hathout et al. 2002). For these reasons, melphalan was used instead of 4-hydroxycyclophosphamide in this study.

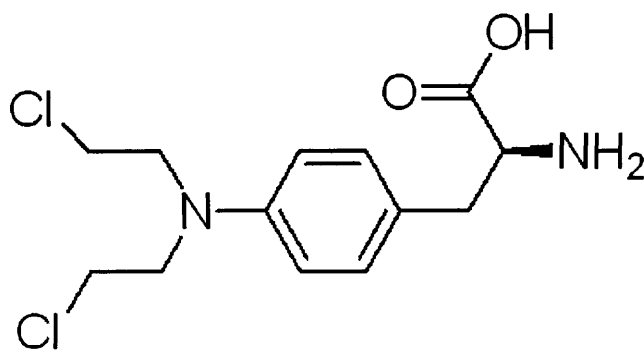


FIGURE 2.2. 4 Structure of melphalan.

Molecular weight 305.20

PACLITAXEL

This is a potent drug that binds to the N-terminal region of β - tubulin, promoting the formation of highly stable microtubules that resist depolymerisation, thus preventing normal cell division and inducing apoptosis. These agents (part of the taxane group) are considered one of the major recent advances in the treatment of breast cancer (Nabholtz & Gligorov 2005).

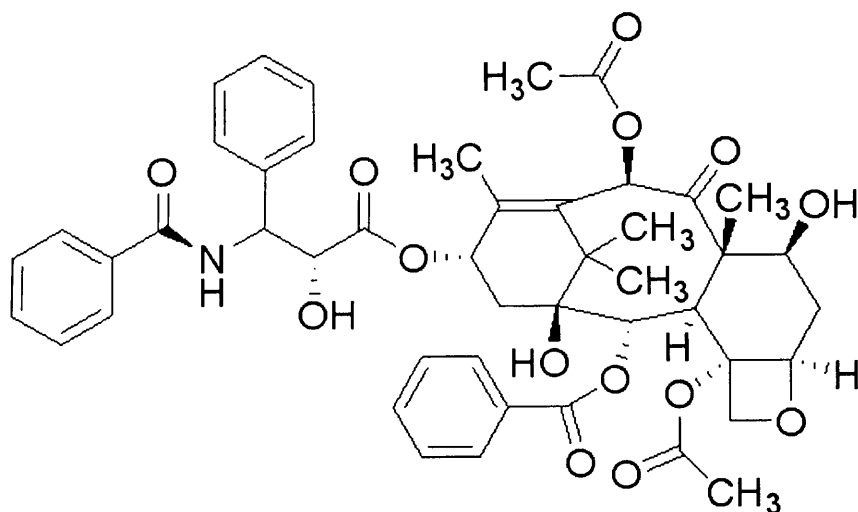


FIGURE 2.2. 5 Structure of paclitaxel
Molecular weight 853.91

CISPLATIN

The mechanism of action of this drug is to form cytotoxic adducts with the DNA dinucleotide, inducing intrastrand crosslinks and apoptosis. It is given usually in combination with other drugs to treat advanced breast cancer refractory to other treatments (Heinemann et al. 2005) or as first-line therapy (Vassilomanolakis et al. 2005).

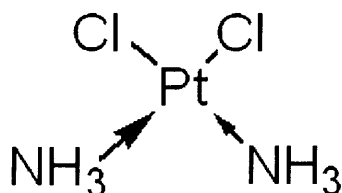


FIGURE 2.2. 6 Structure of cisplatin.
Molecular weight 300.05

2.2.4.4 ASSESSMENT OF CELL INTEGRITY

In this study, the Annexin V – affinity assay was chosen to assess cell integrity by measuring apoptosis and cell death respectively (van Engeland et al. 1998). Annexin V is a calcium-dependent phospholipid-binding protein with high affinity for phosphatidylserine residues on the outer cell membrane. An early, irreversible stage of apoptosis (independent of cell type) involves loss of membrane permeability, resulting in translocation of phosphatidylserine residues from the inner cell membrane (where they are inaccessible to annexin V) to the outer surface. In this way, Annexin V could be used to identify and quantitate the number of cells undergoing apoptosis. In necrosis or cell death, cell membranes are permeable to propidium iodide that stains the whole cell. By using a combination of annexin/PI staining, cells undergoing early to late stage apoptosis and cell death could be quantified.

3. METHODS

3.1 CLINICAL PARAMETERS

Clinical characteristics were obtained from all study subjects, and included:

- Date of birth (age calculated in years)
- Parity
- Menstrual cycle length (days)
- Body mass index (kg/m^2)

For patients only, specific data included:

- Chemotherapy type, schedule, cumulative dosage
- Chemotherapy start and finish date (including length of time from completion of chemotherapy to time of first testing in the case of patients recruited for the first time following chemotherapy)

3.2 BIOCHEMICAL OVARIAN RESERVE TESTS

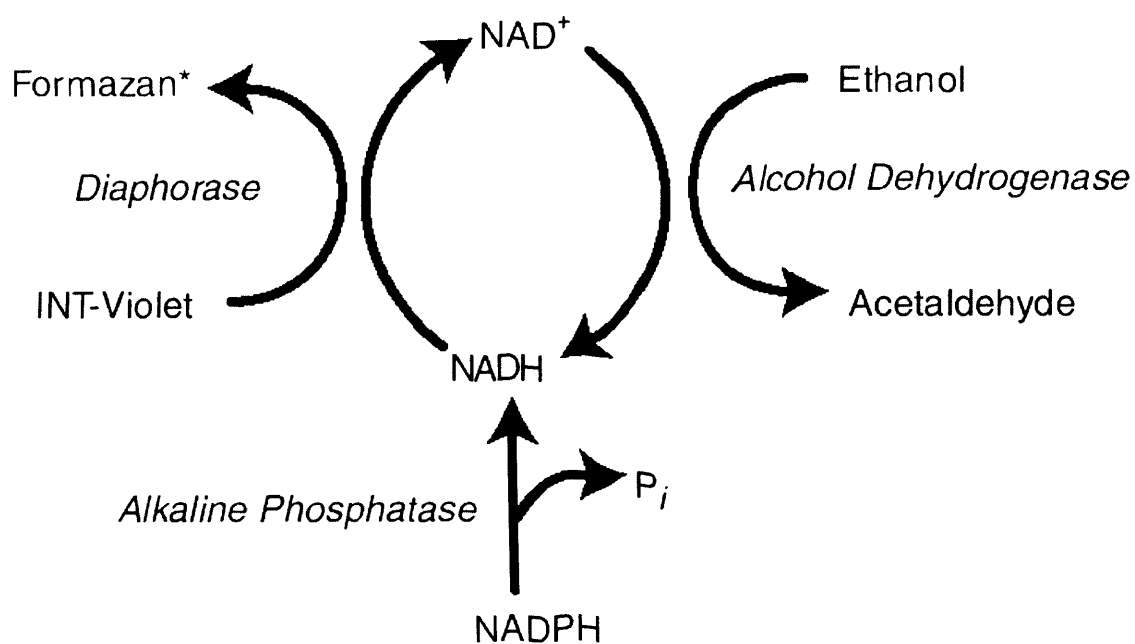
3.2.1 SAMPLE COLLECTION, PREPARATION AND STORAGE

Blood samples (15ml) were obtained from all subjects during the early follicular phase of the menstrual cycle (day 2 – 5). Four timed samples were performed, 15 minutes apart. To minimize discomfort, venesection was performed by inserting an indwelling “butterfly” needle. This allowed timed samples to be taken over the 45-minute time span without the need for repeated needlesticks. Following the last timed sample, the G-test was commenced. This involved administering a gonadotrophin releasing hormone analogue (GnRHa) – Buserelin (Sanofi-Aventis, UK) 1mg subcutaneously. This route was chosen to ensure compliance as opposed to intranasal administration, which would have involved giving 4 separate doses. Administration of the G-test would result in a

transient and reversible ovarian stimulation comparable in magnitude to a physiologic response. This response was quantified after performing a single repeat blood sample approximately 24 hours later. Serum was separated and stored at -20°C for hormone measurements. Assaying each of the 4 samples and determining an average value then determined mean serum concentrations for FSH, LH and oestradiol. This was designed to overcome the effect of pulsatile gonadotrophin release, the frequency of which is increased in the follicular phase (Filicori et al. 1986; McCartney et al. 2002). For the activins, inhibins and AMH, pooled samples were obtained from the 4-timed samples prior to assaying (obtained by mixing equivalent volumes from each timed sample prior to freezing).

3.2.2 INHIBIN A, ACTIVIN A, PRO α C AND FOLLISTATIN

These were all in-house, two-site “sandwich” enzyme-linked immunosorbent assays (ELISA’s), which use monoclonal antibodies for highly specific subunit capture and detection. These antibodies have been developed and the ELISA methods validated by researchers at Oxford Brookes headed by Professor Nigel Groome (Evans LW & Groome 2001). In all these assays, the alkaline phosphatase detection system is used (ELISA Amplification System, Invitrogen Life Technologies, Paisley, UK). In this system, the bound enzyme acts as a substrate whose product initiates a secondary cyclic enzyme reaction, resulting in a coloured product.



*Colored end product

FIGURE 3.2.1 Biochemical basis for ELISA amplification system.

Substrate: Reduced nicotinamide adenine dinucleotide phosphate (NADPH).

The rate of reduction of the tetrazolium salt (INT-Violet) is directly proportional to the concentration of reduced nicotinamide adenine dinucleotide (NADH) originally formed by the enzyme in the bound conjugate.

3.2.2.1 GENERAL ASSAY REQUIREMENTS

These assays require the use of buffers, which are produced in-house as described below:

BUFFERS:

- Inhibin A assay diluent
- Normal ELISA wash buffer
- Manual wash buffer

PREPARATION OF BUFFERS

Inhibin A assay diluent:

Enzyme immunoassay diluent was made using 10% (w/v) bovine serum albumin (BSA), 5% (v/v) mouse serum), 5% (v/v) Triton® X-100, 0.15 M sodium chloride and 0.1% sodium azide made up in 0.1M Tris – HCl buffer at pH 7.5. For example, a 200ml stock solution of inhibin A assay diluent would be made up by adding together 20g BSA, 10ml mouse serum, 10ml Triton® X-100 (Sigma-Aldrich Company Ltd., Gillingham, Dorset SP8 4XT, UK.), 1.75g sodium chloride, 0.2g sodium azide and 18ml 1M Tris HCl buffer with 162ml distilled water. The constituents are then mixed well for at least 30 to 60 minutes, and then filtered through cotton wool. This was then stored in a refrigerator at 4-8 °C.

Normal ELISA wash buffer (X 20 stock):

Stock ELISA wash buffer was made using 0.05M Tris base, 0.15M sodium chloride and 0.05% sodium azide mixed together using distilled water and pH neutralised to pH7.5 using concentrated hydrochloric acid (HCl).

Normal wash buffer was then made up by adding stock buffer to distil water, tween 20 and then stirred well for 30 minutes and stored at room temperature.

3.2.2.2 GENERAL PRINCIPLES

Laboratory safety procedures and protocols were adhered to at all times. White labcoats were worn at all times inside the lab and protective gloves were used whenever samples were handled. A lab-book was used to make a detailed account of all experiments undertaken.

Samples were allowed to defrost at room temperature a couple of hours prior to the assay being performed. Once fully defrosted, samples were properly mixed (Vortex Genie II, Scientific Industries, Inc. Bohemia, NY 11716 USA) prior to use.

3.2.2.3 INHIBIN A

This uses a highly specific monoclonal antibody (E4) to the β A subunit (Groome 1991), which is adsorbed to the microplate for capture, and a monoclonal antibody (R1) (Groome et al. 1990) to α subunit as a Fab alkaline phosphatase conjugate for detection. As such, all forms of inhibin that contain a β A subunit found in human serum and follicular fluid would be measurable by this assay (Groome & Evans 2000).

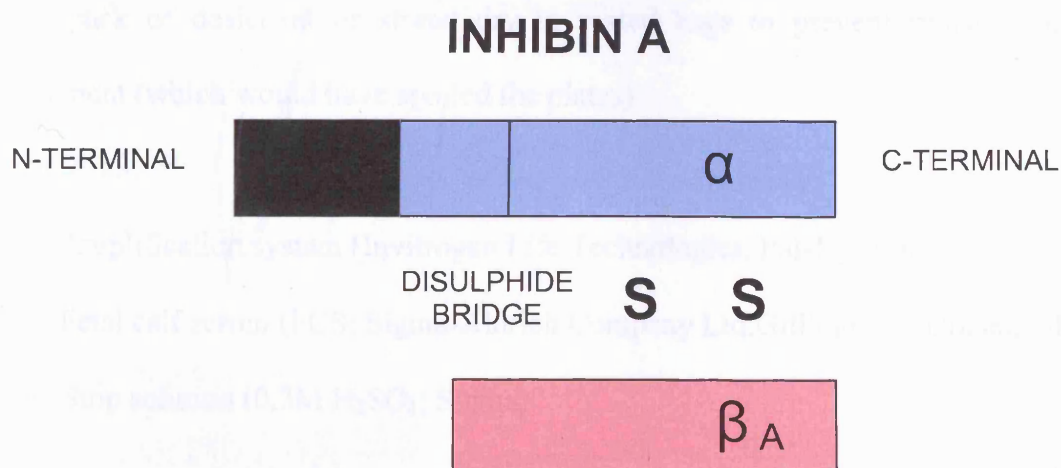


FIGURE 3.2.2 Structure of inhibin A.

ASSAY PROCEDURE

PREPARATION OF PLATES:

Anti β A (E4) coated plates:

Monoclonal antibodies against the β _A subunit of human inhibin (E4; (Groome & Lawrence 1991) were covalently linked to 96-well microtiter plates (Immunoplate, Maxisorb, Nunc, Denmark).

To achieve this, E4 was made in bicarbonate buffer (0.2M) to pH 9.4 (Pierce; Rockford Ill, USA) up to a final concentration of 10 μ g/ml. Once this is prepared, 50 μ l was dispensed into each well. The plate was incubated overnight at room temperature in a moist chamber/environment. After overnight incubation, excess antibody was discarded from the wells and the plate dried by inversion onto paper towelling. A dry coating agent (1:2 dilutions with distilled water) was then added in a volume of 120 μ l/well, and then incubated for 2 hours at room temperature. Following incubation, the excess dry-coating agent was discarded and the plate placed under a flow hood to air dry for a minimum of 4 hours. After this was completed, the plates were foil-wrapped with a pack of desiccant or stored dry in sealed bags to prevent moisture in the environment (which would have spoiled the plates).

REAGENTS:

- Amplification system (Invitrogen Life Technologies, Paisley, UK.)
- Fetal calf serum (FCS; Sigma-Aldrich Company Ltd. Gillingham, Dorset, UK.)
- Stop solution (0.3M H₂SO₄; Sigma)

DAY 1: PREPARATION OF STANDARDS AND SAMPLES

A plate plan was prepared prior to starting the assay.

All samples and standards were made using 2ml flat bottom disposable plastic tubes (Hughes & Hughes Ltd. UK). These were all defrosted and vortexed prior to use.

Human recombinant inhibin A (National Institute for Biological Standards [NIBSC], Potters Bar, Herts UK.) standard was stored at 1ng/ml or 50 pg/50µl (1pg/µl, 200µl aliquots) and used as a reference standard. This was serially diluted in enzyme immunoassay diluent alone or in enzyme immunoassay diluent mixed with an equal volume of pooled human post-menopausal serum (PMS). The top standard was prepared by adding 200µl of FCS (equivalent to 1000pg/ml). A further 7 doubling dilutions were then made by mixing 200µl of FCS with 200µl of the higher standard concentration to give the following range of standards:

25pg/well/50µl (top), 12.5pg/well, 6.25pg/well, 3.13pg/well, 1.56pg/well, 0.78pg/well, 0.39pg/well and 0.195pg/well

The minimum detection limit for this assay was 2 pg/ml (NIBSC, UK.) and the intra- and inter-assay variations were < 6%. Any duplicate showing variability > 10% was repeated.

Samples and standards were then added to the tubes as follows:

- Standards: 125µl to labelled tubes
- Blanks: 125µl FCS
- Samples: 125µl to labelled tubes

Quality controls (QC's): 125µl/tube QC1 = 1:500 human follicular fluid (hFF), QC2 = 1:200 hFF, QC3 = 1:100hFF. Alternatively, quality controls were derived from pooling 6 patient serum samples and 6 control samples respectively.

All samples, standards and quality controls were diluted (1:1) using 125µl of inhibin A assay diluent.

The sensitivity of the assay can be improved by a pre-assay oxidation step (Muttukrishna et al. 1994). This was achieved by adding 50µl of 10% (v/v) hydrogen peroxide (H₂O₂; Sigma) to all tubes (available from stock at 30%, stored at 4° C). The tubes were then mixed thoroughly and incubated for 30 minutes at room temperature before transferring 100µl aliquots (blank/standard/sample/QC) to the E4-coated plates. Following transfer of the oxidised standards and samples to E-4 coated plates; the plates are then covered with a lid and incubated overnight in a moist box at 4°C.

DAY2:

After overnight incubation, the samples are discarded and the plate washed (10 cycles) using an automated microplate washer (Labsystems Wellwash 4 Mk 2, Finland). Following this, 50µl of alkaline phosphate-conjugated anti-alpha subunit antibody (1:200 dilutions in assay diluent, R1-Alkphos) is added to each well. After incubating for 2 hours in a moist box at room temperature on a microplate shaker (Labsystems Wellmix, Finland), plates were washed extensively (17 cycles), with the first 15 cycles being done using an automatic microplate washer. The final 2 washes are done by using the manual wash buffer (250µl/well) and incubating on the plate shaker for 10 minutes before repeating.

Quantification of bound alkaline phosphatase was made using a commercially available enzyme immunoassay amplification kit (Invitrogen Life Technologies, Paisley, UK.), which was used according to the supplier's instructions. To minimise the risk of temperature-dependent variation in signal generation, both incubation steps were performed at ambient temperature. After incubation with 'kit substrate solution' (50µl/well) for 1 hour on a microplate shaker, 50µl/well of 'kit amplifier solution' was added to each well. The amplification reaction was stopped when before blank wells began to show significant colour development by adding 50µl of 0.3M sulphuric acid

(normally stopped after ~ 1 h). Absorbance at 492nm was read on an enzyme immunoassay plate reader (Dynex Technologies Inc., VA, USA) and data processed by immunoassay curve-fitting software (Dynex Technologies Inc., VA, USA).

3.2.2.4 ACTIVIN A

This assay uses a monoclonal antibody E4 to the β_A subunit attached to a microplate for capture and biotinylated E4 and streptavidin alkaline phosphatase for detection (Knight, Muttukrishna, & Groome 1996). The activins are structurally and functionally related to the inhibins. While inhibin-A and B are heterodimers consisting of an alpha subunit and a beta A or beta B subunit linked by disulphide bonds, activins are dimers of beta subunits. This assay measures total activin A (follistatin bound and unbound activin A). The detection limit of this assay for human recombinant activin A was 50 pg/ml. Intra- and inter-assay variations were 8.5 and 9.8% respectively (Genentech, San Francisco, CA, USA). Duplicates, which varied $> 10\%$, were repeated.

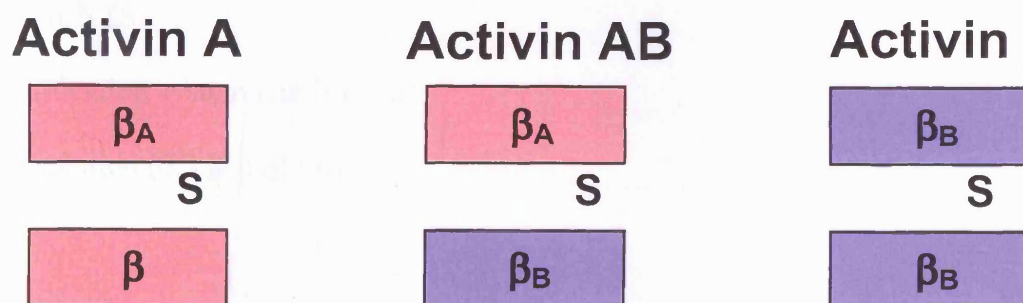


FIGURE 4.2.3 Structural relationship of the activins.

Structural relationship of the activins, which are dimers of the β subunits of the inhibins linked by disulphide bonds (S).

ASSAY PROCEDURE

REQUIREMENTS:

BUFFERS:

- Sample buffer: PBS (phosphate buffered saline) + 10% BSA (bovine serum albumin)
 - Inhibin A assay diluent
 - Normal ELISA wash buffer
 - Manual wash buffer
- (Previously described section 4.2.2.1)

Preparation of sample buffer (PBS + 10%BSA):

Sample buffer (100ml) was made by dissolving 1PBS tablet in 100ml (25mmol) distilled water. This was added to 10g BSA and 100mg sodium azide and the entire solution mixed properly on the magnetic stirrer (Genesis hotplate stirrer, Jencons Scientific Ltd, Bedfordshire, UK).

REAGENTS:

Amplification system (Invitrogen)

Stop solution (0.3 M H₂SO₄)

PLATES:

Anti β a (E4) coated plates (previously described in section 3.2.2.1)

DAY 1: PREPARATION OF STANDARDS AND SAMPLES

A plate plan was prepared prior to starting the assay.

All standards, samples and controls were defrosted and mixed prior to use. Heat resistant eppendorf tubes (Sarstedt, Aktlengesellschaft & Co. Germany) were used for preparing all samples and standards. The activin A standard consisted of affinity purified human follicular fluid which was standardised against human recombinant Activin A (Genentech, San Francisco USA). The standard was prepared beforehand by adding 250ng activin A to 5ml PBS+1% BSA and 0.1% azide and mixed well. This provided a reconstituted concentration of 50ng/ml (100µl aliquots), which is stored at -20°C.

To prepare the top standard concentration, 400µl of PBS + 10%BSA were added to the stored standard and mixed well. This provided a top standard concentration of 500pg/well/50µl (10ng/ml). A further 7 doubling dilutions were made by mixing 200µl of PBS+10%BSA with 200 µl of the higher standard concentration to give the following range of standards:

250pg/well/50µl, 125 pg/well/50µl, 62.5 pg/well/50µl, 31.3 pg/well/50µl, 15.6 pg/well/50µl, 7.8 pg/well/50µl and 3.9 pg/well/50µl.

Blank samples were made up of PBS+10%BSA buffer only. Quality controls (QC's) were derived from third trimester pregnancy serum as follows: QC1 = 1:8 dilution, QC2 = 1:4 dilution and QC3 = 1:2 dilution as well as a representative sample of 6 patients.

Volumes (175µl) of diluted samples and standards were transferred to 1.5ml Eppendorf tubes and mixed with an equal volume (175µl) of 15% (wt/vol) sodium dodecyl sulphate (SDS). This was prepared by mixing together (by inversion) 1.5g SDS with 10ml-distilled water warmed to about 60°C. The tubes were then incubated for 10 minutes at 85-90°C in a waterbath (Grant Instruments, Cambridge, UK). This step is

done to overcome interference caused by follistatin seen in previous assays (Wong et al. 1993) by dissociating follistatin, thus allowing measurement of total activin A. After cooling for about 5 minutes, 30µl of hydrogen peroxide solution was added to all tubes (30%, vol/vol; Sigma Chemical Co., St. Louis MO, USA). The tubes were mixed well on the whorly mixer and incubated for a further 10 minutes at room temperature.

The E4 dry coated plate was prepared by first adding 25µl/well inhibin A assay diluent using an electronic multi-channel dispenser (Genex Alpha, Genex Laboratory Products, Northampton UK). Duplicate 100µl aliquots of standards/samples were then transferred to the plate and then mixed on a microplate shaker for a few minutes. Biotynilated E4 (25µl) was then added to each well (dilute 1:100 using inhibin A diluent; stock ~ 0.14mg/ml). The plate was then incubated overnight in a humidified box at 4 °C.

DAY 2:

The plate was washed 10 times using the automatic plate washer with the ELISA wash buffer, and then dried by inversion onto paper towelling. Alkaline phosphatase-conjugated extravidin (1:10000, vol/vol; Sigma) was then added (50µl/well) using a manual multichannel dispenser (Scientific Laboratory Supplies, Nottingham, UK). The plate was then incubated for 2 hours at room temperature in a moist box on a plate shaker, then washed for 15 cycles using the automated plate washer and dried on paper towelling. This was followed by 2 manual washes using the manual wash buffer. The latter was achieved by adding 250µl/well manual wash buffer and incubating on the plate shaker for 5 minutes before repeating.

Bound alkaline phosphatase was then quantified using a commercially available amplification kit (Invitrogen Life Sciences). Substrate was added (50µl/well) and the plate incubated for 1 hour on a moist box placed on a shaker. Amplifier was then added

(50µl/well) and the reaction stopped using 50µl/well stop solution when the blanks started to develop colour. Absorbance was then read at 490nm.

3.2.2.5 FOLLISTATIN

Follistatin is a monomeric glycosylated polypeptide chain. There are 2 mature mammalian follistatin (FS) forms: FS 288 and FS 315, although the latter is only 10% cross-reactive. This ELISA uses a pair of mouse monoclonal antibodies to FS 288: 29/9 as the capture antibody and the Fab' fragment of monoclonal antibody 17/2 for detection. Use is made of a dissociating diluent to disrupt activin/follistatin complexes so that the assay measures 'total' human follistatin (Evans, Muttukrishna, & Groome 1998).

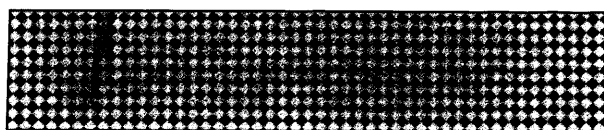


FIGURE 3.2.4 Structure of follistatin.

ASSAY PROCEDURE

REQUIREMENTS:

BUFFERS:

Follistatin sample buffer (dissociating solution):

1L PBS is used to make 1L buffer, to which the following were added and mixed (in this order): 35g sodium deoxycholate (3.5%w/v); 34g Tween 20 (3.4% w/v); 10g BSA (1%w/v); 50ml mouse serum (5%w/v); 1g sodium azide (0.1% w/v). After mixing the buffer was stored at 4°C.

Follistatin assay buffer (Tris conjugate buffer):

500ml of 25mM Tris HCl (pH 7.5) is used to make 500ml assay buffer, to which the following were added and mixed (in order): 5g BSA (1%w/v); 4.4g NaCl (0.15M); 2.5ml Tween 20 (0.5% v/v); 0.5g sodium azide (0.1%w/v). After mixing the buffer was stored at 4°C.

Normal ELISA wash buffer: (previously described in section 3.2.2.1)

PLATES:

29/9-coated 96-well plates (ready to use)

REAGENTS:

Amplification system (Invitrogen Life Sciences)

Stop solution (0.3M H₂SO₄; Sigma)

DAY 1: PREPARATION OF STANDARDS AND SAMPLES

A plate plan was prepared prior to starting the assay.

All samples and standards were made using 2ml flat bottom disposable plastic tubes, after defrosting and mixing. The standard (freeze-dried, immunopurified human follistatin) was reconstituted in 10.3mls dissociating solution giving a top standard of 2500pg/ml. Further dilutions were made to provide the following standard dilutions:

2500pg/ml; 1250pg/ml; 625pg/ml; 313pg/ml; 156pg/ml; 78pg/ml; 39pg/ml and 19.5pg/ml.

Serum samples and QC's were diluted 1:3 in dissociating solution. The purpose of the dissociating solution is to disrupt activin-follistatin complexes, thus enabling only total follistatin to be measured (Groome et al. 2001b). The sensitivity of this assay was 20pg/ml, and the intra- and inter-assay variations were less than 10% respectively. Duplicates above 10% were repeated.

Duplicate 50µl amounts of standards, samples, blanks (sample buffer) and QC's are added to the wells on the plate, which were then sealed and incubated overnight stationary at room temperature in a sealed, moist box.

DAY 2:

The plate was washed 10 times with ELISA wash buffer in the automated plate washer. To each well 50µl of approximately 1µl/ml solution of the Fab fragment of clone 17/2 (attached to alkaline phosphatase) was added, diluted in Tris conjugate buffer. After 2-hour incubation in a moist box at room temperature, the plate was again washed thoroughly 10 times and dried by inversion unto paper towelling. Alkaline phosphatase substrate (50µl) was added to each well and the plate-incubated (stationary at room temperature) for 24 hours. Amplifier solution (50µl) was then added to each well and

colour allowed to develop. The reaction was stopped using stop solution (50µl) to each well when the blanks developed colour. The absorbance was read at 490nm.

3.2.2.6 PRO α C

This assay uses a monoclonal antibody to the pro region of the human inhibin α subunit for capture and the same R1 Fab alkaline phosphatase conjugate as in the inhibin B ELISA's for detection (Groome et al. 1995). This assay is expected to measure precursor forms of both inhibin A and inhibin B (shown below) carrying the full-length α subunit as well as free forms of the α subunit such as pro α C and pro α N α C. Since these free forms are present in excess over dimeric inhibins in most body fluids in practice, the assay is measuring higher molecular weight dimeric inhibins and non-bioactive monomeric α subunits. The detection limit for this assay was approximately 2pg/ml, and the intra- and inter-assay variations <15%.

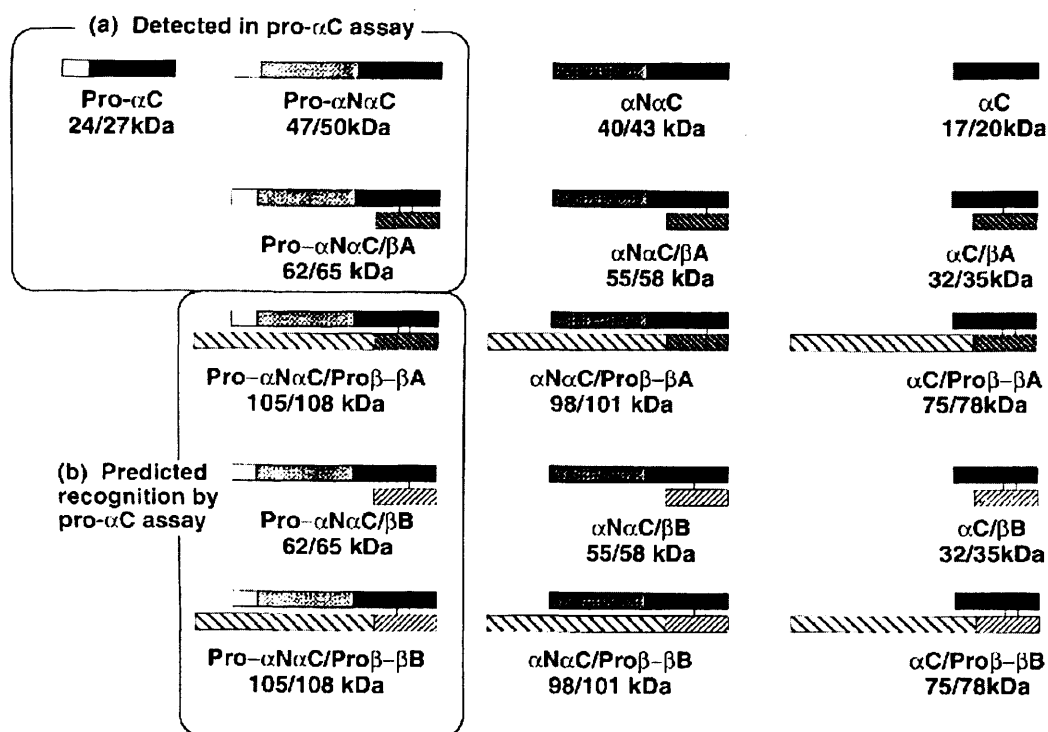


FIGURE 3.2.5 Diagrammatic representation of inhibin precursor forms measurable by this assay. (Adapted from Oxford Brookes website <http://www.brookes.ac.uk/inhibin/>).

ASSAY PROCEDURE

REQUIREMENTS:

BUFFERS:

- Inhibin A assay diluent
 - Normal ELISA wash buffer
 - Manual wash buffer
- } (previously described in section 3.2.2.1)

REAGENTS:

Amplification system (Invitrogen Life Sciences)

Fetal calf serum (FCS; Sigma)

Stop solution (0.3M H₂SO₄; Sigma)

PLATE:

96 well Pro α C dry –coated plate (ready to use)

DAY 1: PREPARATION OF STANDARDS AND SAMPLES

A plate plan was prepared prior to starting the assay.

All samples and standards were made using 2ml flat bottom disposable plastic tubes.

The standards (Pro α C peptide kindly provided by Professor Nigel Groome, Oxford Brookes, UK.), which were stored at 2ng/ml stock, were serially diluted in enzyme immunoassay diluent alone (1:1) to obtain the top standard concentration (50pg/well/50 μ l). A further 7 doubling dilutions were made by mixing 150 μ l (for 1 plate) of assay diluent with 150 μ l of the top standard concentration to give the following range of standards:

200pg/ml (top), 100pg/ml, 50pg/ml, 25pg/ml, 12.5pg/ml, 6.25 pg/ml, 3.13pg/ml and 1.56pg/ml.

All samples were defrosted and mixed prior to assaying. Blank wells comprised 100µl assay diluent only. Quality controls comprised 100µl human follicular fluid (hFF) available in dilutions of 1:5000, 1:2500 and 1:1250 with assay diluent. Pooling 6 samples and performing dilutions of 1:2, 1:4 and 1:8 provided additional controls. To each labelled well, 100µl of blanks, standards and samples were added in duplicate. The plate was then incubated in a moist box on a microplate shaker.

DAY 2:

Following overnight incubation, the plate was washed 10 times using an automated plate washer, and then dried by inversion on paper towelling. To each well, 50µl of 1:200 alkaline phosphatase conjugated R1 Fab was added. The plate was then incubated for 2 hours in a moist box. After this, the plate was washed a further 17 times: the first 15 washes using the automated plate washer and the final 2 times done manually using 250µl/well of manual wash buffer. The plate was then dried by inversion on paper towelling.

Substrate was prepared 10 minutes prior to adding to all wells (50µl/well) in the same sequence that the R1 Fab was added. The plate was then incubated in a moist box for 1 hour. Amplifier was then added (50 µl/well) to each well in the same sequence as the substrate. The plate was then mixed for a few minutes on the automated plate shaker and the reactions stopped using 50µl/well stop solution before the blanks develop colour (normally 10-20 minutes). The absorbance was read at 492nm.

3.2.3 INHIBIN B

This assay was first developed in 1996 where it was used to study serum inhibin B concentrations throughout the human menstrual cycle (Groome et al. 1996). It uses a monoclonal antibody to the β B subunit adsorbed to a microplate for capture and a Fab conjugate of monoclonal antibody R1 attached to alkaline phosphatase for detection. This assay is predicted to measure a range of size isoforms of inhibin B found in human serum and follicular fluids (Groome et al. 2000).

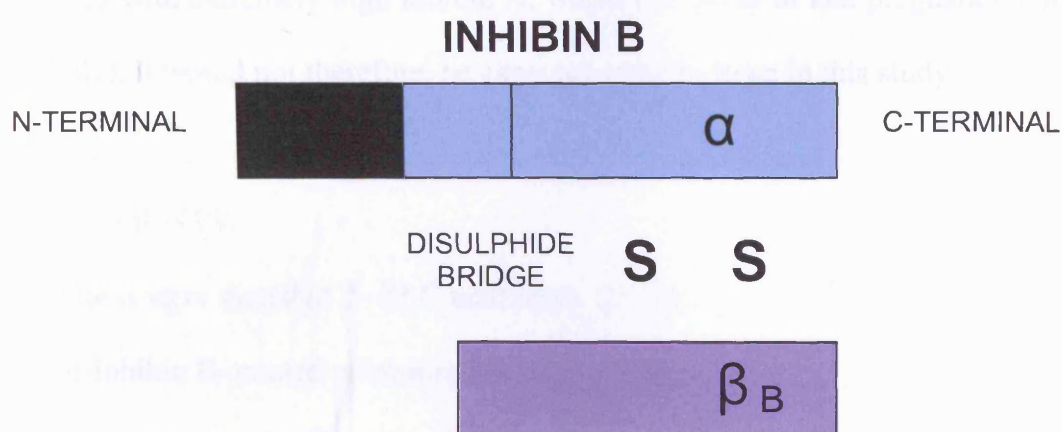


FIGURE 3.2.6 Structure of inhibin B. **S** = disulphide bonds

ASSAY PRINCIPLE

A commercial kit – the DSL-10-84100 ACTIVE[®] Inhibin B Enzyme-Linked Immunosorbent Assay (ELISA) Kit (Diagnostic Systems laboratories, Inc. Texas, USA.) was used for all inhibin B assays performed in this study. In this assay, Standards, Controls and unknown serum samples were incubated overnight in microtitration wells, which had been coated with anti-inhibin β_B subunit antibody. After incubation and washing, the wells were incubated with biotinylated anti-inhibin α -subunit detection antibody and the immunoreactions monitored by subsequent addition of streptavidin labelled with enzyme horseradish peroxidase (HRP). After a third incubation and washing step (the washing steps were crucial to the performance of the assay), the wells were incubated with the substrate tetramethylbenzidine (TMB).

An acidic stopping solution was then added and the degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 450 and 620nm. The absorbance measured was directly proportional to the concentration of inhibin B present. The sensitivity of this assay was approximately 10 pg/ml, and the intra- and inter-assay variations <10%.

This assay has a small cross-reaction with inhibin A (0.5%). The cross-reaction is only an issue when attempting to measure very low concentrations of serum inhibin B in samples with extremely high inhibin A, which can occur in late pregnancy (Groome et al. 2001). It would not therefore, be expected to be an issue in this study.

REQUIREMENTS:

All products were stored at 2 - 8° C until ready for use.

1. Anti-Inhibin B- coated microtitration strips (plate)
2. Inhibin B Standard A/Sample diluent (2ml)
3. Inhibin B Standards: Each vial contained 1ml each containing concentrations of approximately 10, 30, 100, 250, 500, and 1000pg/ml dimeric inhibin B in fetal bovine serum (FBS).
4. Inhibin B Controls: Each vial contained 1ml each containing low and high concentrations of dimeric inhibin B in FBS.
5. Inhibin B Sample Buffer A
6. Inhibin B Sample Buffer B
7. Inhibin B Antibody-Biotin Conjugate
8. Streptavidin-Enzyme Conjugate
9. TMB Chromogen Solution
10. Wash Concentrate: 100ml bottle containing buffered saline with a non-ionic detergent. The wash solution was prepared by adding 900ml of distilled water.

ASSAY PROCEDURE

DAY 1:

A plate plan was prepared prior to starting the assay.

Using a pipettor (Gilson S.A.S. France) 50µl of standards, controls and samples were transferred into the appropriate microtitration wells. This was followed by adding 25µl of Inhibin B sample buffer A and B respectively to each well using a semi-automatic dispenser (Gilson, France). The plate was then incubated in a moist box, overnight at room temperature on the workbench (static incubation).

DAY2:

Following overnight incubation (14-18 hours), the wells were washed five times with distilled water using an automatic microplate washer (Labsystems Wellwash 4 Mk2, Finland), and the plate blotted dry by inversion on a paper towel. The next step was to add 50µl of biotinylated anti α subunit to all wells in the plate, which was then incubated for one and a half hours at room temperature in a moist box. Using a semi-automatic dispenser, 50µl of the streptavidin-enzyme conjugate was added to each well. The plate was then covered and incubated for 20 minutes at room temperature. A further 2 washing cycles followed using the wash solution. In the first, the plate was washed 6 times using an automatic microplate washer. After the last washing cycle, the washer was stopped and the wells soaked in the wash solution for 15 minutes. The second wash cycle was then commenced for a total of 6 cycles. After the last cycle, the plate was then dried by inversion on paper towelling.

To each well, 100µl of TMB chromagen solution was added using a semi-automatic dispenser. The plate was covered with foil (to avoid direct sunlight) and incubated for 15-30 minutes at room temperature. Once optimal colour development had been achieved, 100µl of stopping solution was added to each well using a semi-automatic

dispenser. Absorbance at 450nm was read on an enzyme immunoassay plate reader (Dynex Technologies Inc., VA, USA) within 30 minutes of adding the stopping solution and the data processed by immunoassay curve-fitting software (Dynex Technologies Inc., VA, USA).

3.2.4 ANTI MÜLLERIAN HORMONE (AMH)

A commercial kit – the Immunotech AMH enzyme immunoassay (Immunotech SA Marseille, France) was used for quantification of AMH in all serum samples used in this study.

ASSAY PRINCIPLE

This ELISA was a two-step sandwich type assay. In the first step, a monoclonal antibody attached to the microtiterplate was used for capture. In the second step a biotinylated monoclonal antibody mixed with streptavidin alkaline phosphatase was used for detection. The sensitivity of this assay was 0.7pM, and the inter- and intra-assay variations 14.2% and 12.3% respectively. Duplicates, which varied > 10%, were repeated.

REAGENTS:

The kit including the reagents was stored at 2-8°C until ready for use, when it is allowed to come to room temperature. The expiry date was checked prior to use.

1. Anti-AMH antibody-coated plate (ready to use)
2. Reaction buffer (ready to use)
3. Biotinylated monoclonal antibody (for reconstitution)
4. AMH calibrator (for reconstitution)

5. Calibrator 0 (human serum)
6. Wash solution (for dilution)
7. Streptavidin-HRP (ready to use)
8. Substrate (ready to use)
9. Stop solution (2N H₂SO₄)

ASSAY PROCEDURE

PREPARATION OF STANDARDS AND SAMPLES

A plate plan was prepared prior to starting the assay.

All samples and standards were defrosted and mixed prior to use, and prepared using 2ml flat bottom disposable plastic tubes.

The 1500pM (top) and 0pM (bottom) calibrators were reconstituted using distilled water and used to make a fresh dilution series as follows:

Calibrator concentration (pM)	AMH	Calibrator 0 (μL)
150	50μL of 1500 pM calibrator	450
81	200μL of 150pM calibrator	170
27	100μL of 81pM calibrator	200
9	100μL of 27pM calibrator	200
3	100μL of 9pM calibrator	200
0	-	200

TABLE 3.2.1 Preparation of AMH calibrators.

PROCEDURE:

25µL of samples and standards were added to each well in duplicate according to the plate plan. To each well, 100µL of reaction buffer was added. The plate was then incubated at room temperature (18-25 °C) on the plate shaker for 2 hours. After this, the plate was washed manually a total of 5 times.

In the next step, 50µL of biotinylated antibody and 50µL of streptavidin-HRP were added to the wells. The plate was then incubated for 30 minutes at room temperature on the plate shaker. Following this incubation, the plate was again washed manually 5 times.

In the final step, 100µL of substrate was added to each well and the plate incubated at room temperature for 30 minutes on the shaker and colour allowed to develop. The reaction was stopped using stop solution (50µl) to each well when the blanks developed colour. The absorbance was read at 490nm using an enzyme immunoassay plate reader (Dynex Technologies Inc.) and data processed by immunoassay curve-fitting software (Dynex Technologies Inc.).

3.2.5 FOLLICLE STIMULATING HORMONE (FSH)

A commercial kit – the IBL FSH EIA (IBL Immuno-Biological Laboratories Hamburg, Germany) was used for quantification of FSH in all serum samples used in this study.

ASSAY PRINCIPLE

FSH is a glycoprotein hormone, which is part of a family of cysteine rich proteins consisting of an alpha (α) and beta (β) subunit. Like other glycoprotein hormones such as Luteinising hormone (LH), Thyroid stimulating hormone (TSH) and Human chorionic gonadotrophin (HCG), the alpha subunit is common to each of these

hormones for a given species, while the β subunit is assumed to confer hormone specificity.

This was a solid phase ELISA based on the sandwich principle. The microtiter wells were coated with a monoclonal antibody directed towards a unique antigenic site on a β -FSH molecule. An aliquot of sample serum containing endogenous FSH was incubated in the coated well with enzyme conjugate, which is an anti- α -FSH antiserum conjugated with horseradish peroxidase. The amount of bound peroxidase was therefore proportional to the amount of FSH in the sample. The sensitivity of this assay was 2mIU/ml, and the intra- and inter-assay variations were less than 10%. Duplicates which varied > 10% were repeated.

REAGENTS:

The kit including the reagents was stored at 2-8°C until ready for use, when it is allowed to come to room temperature. The expiry date was checked prior to use.

1. Microtiter plate coated with anti-FSH monoclonal antibody
2. Enzyme conjugate (ready to use)
3. FSH standards: lyophilised, for reconstitution in distilled water
4. Substrate: TMB (ready to use)
5. Stop solution: 0.5M H₂SO₄

PREPARATION OF STANDARDS AND SAMPLES

A plate plan was prepared prior to starting the assay.

All samples and standards were made using 2ml flat bottom disposable plastic tubes (Hughes and Hughes Ltd.) after defrosting and mixing. Once the assay had started, it was important that all steps should be completed without interruption. Absorbance is a function of the incubation time and temperature, and as such manual pipetting of all standards, samples and controls were completed within 10 minutes.

Standards were prepared by reconstituting the lyophilised contents of the standard vials with 1ml-distilled water to create the following range of dilutions:

Concentration (mIU/ml): 0 5 10 20 50 100

Quality controls were provided by pooled samples as previously described.

ASSAY PROCEDURE:

To appropriate wells were added 25µL of standards, controls and samples. Following this step, 100µL of enzyme conjugate were added to each well. The plate was then mixed thoroughly for 10 seconds and then incubated on a microplate shaker for 30 minutes at room temperature (18-24 °C). Following the incubation, the plate was manually washed with distilled water and the plate dried by inversion onto paper towelling. Substrate was then added at timed intervals to each well in 100µL volumes, and the plate covered in foil and incubated for 10 minutes at room temperature to allow colour to develop. The enzymatic reaction was stopped by adding 50µL of stop solution to each well in a similar timed interval as described above. The absorbance was read at 450nm using an enzyme immunoassay plate reader within 10 minutes of adding the stop solution (Dynex Technologies Inc), and data processed by immunoassay curve-fitting software (Dynex Technologies Inc).

3.2.6 LUTEINISING HORMONE (LH)

A commercial kit – the IBL LH EIA (IBL Immuno-Biological Laboratories Hamburg, Germany) was used for quantification of FSH in all serum samples used in this study.

ASSAY PRINCIPLE

Human luteinizing hormone is a glycoprotein, which consists of 2 polypeptide chains called α and β . The α - subunit is identical to the α - subunits of other human glycoprotein hormones such as FSH, HCG and TSH. The β - subunit confers specificity and the immunological behaviour of the hormone.

The microtiter wells of the plate were coated with a monoclonal antibody directed towards a unique antigenic site on a β -LH molecule. An aliquot of sample serum containing endogenous LH was incubated in the coated well with enzyme conjugate, which is an anti- α -LH antiserum conjugated with horseradish peroxidase. The amount of bound peroxidase was therefore proportional to the amount of LH in the sample. After addition of substrate, the intensity of colour developed was proportional to the concentration of LH in the serum. The sensitivity of this assay was 2mIU/ml, and the intra- and inter-assay variations less than 7%. Duplicates >10% were repeated.

REAGENTS:

The kit including the reagents was stored at 2-8°C until ready for use, and then allowed to come to room temperature when ready for use after the expiry date was checked.

1. Microtiter plate coated with anti-LH monoclonal antibody
2. Enzyme conjugate (ready to use)
3. LH standards: lyophilised, for reconstitution in distilled water
4. Substrate: contains tetramethylbenzidine (TMB; ready to use)
5. Stop solution: 0.5M H₂SO₄

ASSAY PROCEDURE

PREPARATION OF STANDARDS AND SAMPLES

A plate plan was prepared prior to starting the assay.

All samples and standards were made using 2ml flat bottom disposable plastic tubes after the samples were defrosted and mixed. Once the assay had started, all steps had to be completed without interruption within 10 minutes for the same reasons described earlier for FSH. Standards were prepared by reconstituting the lyophilised contents of the standard vials with 1ml-distilled water to create the following range of dilutions:

Concentration (mIU/ml): 0 10 20 40 100 200

PROCEDURE:

To appropriate wells were added 25µL of standards, controls and samples. Following this step, 100µL of enzyme conjugate was added to each well. The plate was then mixed thoroughly for 10 seconds and then incubated on a microplate shaker for 30 minutes at room temperature (18-24 °C). The plate was then manually washed with distilled water and the plate dried by inversion onto paper towelling. Substrate was added at timed intervals to each well in 100µL volumes, and the plate covered in foil and incubated for 10 minutes at room temperature to allow colour to develop. The enzymatic reaction was then stopped by adding 50µL of stop solution to each well in a similar timed interval as described above. The absorbance was then read at 450nm using an enzyme immunoassay plate reader within 10 minutes of adding the stop solution.

3.2.7 17 BETA-OESTRADIOL

A commercial kit – the IBL 17 beta-Estradiol ELISA (IBL Immuno-Biological Laboratories Hamburg, Germany) was used for quantification of 17 β -Oestradiol in all serum samples used in this study.

ASSAY PRINCIPLE

Oestradiol (E2) is a C18 steroid hormone with a phenolic A ring.

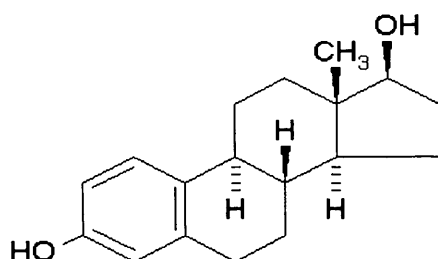


FIGURE 3.2.7 Structure of 17 β -Oestradiol.

Molecular formula: C₁₈H₂₄O₂

This was a solid phase ELISA, based on the principle of competitive binding. The microtiter wells were coated with an antibody, which was directed towards a unique antigenic site on the E2 molecule. Each sample contained endogenous E2, which competed with an E2 horseradish peroxidase conjugate for binding to the coated antibody on the plate. Therefore the amount of bound peroxidase conjugate was inversely proportionate to the concentration of E2 in the sample. After addition of the substrate, the intensity of the colour developed was also inversely proportional to the concentration of E2 in the sample. The sensitivity of this assay was 9.7 pg/ml, and the inter- and intra- assay variations less than 10%. Duplicates samples, which varied > 10%, were repeated.

REAGENTS:

All reagents were stored at 2-8 °C until opened for use (after checking the expiry date) when it was allowed to come to room temperature, and included:

1. Microtiter plate (96 well) coated with anti-E2 polyclonal rabbit antibody
2. Standards (0-6); 7 vials (ready to use)

Concentrations (pg/ml):

0 25 100 250 500 1000 2000

3. Enzyme conjugate: E2 conjugated to horseradish peroxidase (ready to use)
4. Substrate: TMB (ready to use)
5. Stop solution: 0.5M H₂SO₄ (ready to use)
6. Wash solution: 40X concentrated

ASSAY PROCEDURE

A plate plan was prepared prior to starting the assay.

All reagents were ready to use except for the wash buffer. This was prepared by making up the wash buffer (30ml) to a final volume of 1200ml using deionised water.

Once the test had started, all steps had to be completed without interruption, as the absorbance is a generally a linear function of the incubation time and temperature.

To appropriate wells, 25 µL of each standard, sample and control were added using a micropipette dispenser. Following this, 200 µL of enzyme conjugate was added to all wells. The plate was then incubated on an orbital plate shaker at room temperature for 120 minutes (without covering the plate), following which the wells were washed thoroughly (400µL/well) three times using the diluted wash solution. The plate was then dried by inversion onto paper towelling.

In the final step, 100µL of substrate was added to each well. The plate was again incubated at room temperature and colour was allowed to develop. After 15 minutes, the enzymatic reaction was stopped by adding 50 µL of stop solution to each well. Within 10 minutes, the absorbance was read at 450nm.

3.3 BIOPHYSICAL OVARIAN RESERVE TESTS

3.3.1 INTRODUCTION

Ultrasound examination was performed on the same day that blood samples were taken (before ovarian stimulation). Inter-observer variability was minimised by having all ultrasound scans performed by a single investigator with expert ability in assessing these parameters (Conway et al. 1996). An Acuson 128 XP4 (Acuson USA) with a 2.5-4 MHz-vector probe (transabdominal) and a 5-7.5mHz EC-7 (transvaginal) probe were used to perform the scans.

3.3.2 ANTRAL FOLLICLE COUNT

Round or oval shaped echo-free structures from 2mm to 10mm within the ovaries were considered to be follicles. Total AFC was the sum of antral follicles measured from both ovaries.

3.3.3 OVARIAN VOLUME

Ovarian volume was calculated for each ovary using the prolate ellipsoid formula:

Ovarian volume = $D1 \times D2 \times D3 \times \pi/6$, where D1, D2 and D3 were the maximal perpendicular diameters of the ovary.

3.3.4 OVARIAN STROMAL BLOOD FLOW (OSBF)

Ovarian stromal blood flow velocity waveforms were obtained by placing the Doppler gate over the ovarian stroma which had the highest achievable signals, ensuring that no arteries near the surface of the ovary were measured. Peak systolic velocity and pulsatility indices from both ovaries were then combined to provide the Mean Pulsatility Index (MPI) and Mean Peak Systolic velocity (MPS) respectively.

3.3.5 UTERINE DIMENSIONS

Uterine cross-sectional area (cm^2) and endometrial thickness (mm) were calculated by examining the uterus in the sagittal plane.

3.4 IN VITRO STUDY

3.4.1 INTRODUCTION

The study design for the invitro study was discussed in chapter 3. Culture of the breast cell line was carried out at the Ludwig Institute of Cancer Research under the supervision of Professor Mike O'Hare. Culture of granulosa cells was performed at the Centre for Reproductive Science Laboratory of University College London under the supervision of Dr Shanthi Muttukrishna.

3.4.2 BREAST CELL CULTURE

3.4.2.1 ESSENTIAL SKILLS

Before the actual experiment was started, essential cell culture techniques had to be learnt. This was initiated at the time the MCF-7 cell line was thawed and continued for up to one month before the actual experiments were carried out. These skills are listed below and are described in more detail as procedures are explained:

- Switching on the Laminar flow cabinet (LFC), sterilisation with 70% ethanol and ensuring adequate layout.
- Aseptic technique under the LFC, including handling and accurate transfer of fluids with the pipettes and sterilising the LFC after use.
- Preparing medium for use including equilibrating the medium in the flasks with CO₂ using Pasteur pipettes.
- Use of the incubator.
- Feeding cultures and monitoring for contamination with the inverted microscope.
- Counting cells using a haemocytometer and assessing viability using trypan blue.

- Preparation of stock medium, EDTA/PBS and antibiotics. All reagents were usually stored in the refrigerator (4-8 °C) with the exception of EDTA/PBS, which could be stored at room temperature.
- Subculture of established cell lines including accurate determination of seeding density.
- Writing all information in a lab note book and updating it everyday.

3.4.2.2 THAWING THE CELL LINE

MCF-7 human mammary carcinoma cells (Soule et al. 1973b) frozen in ampoules containing 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen was used for the experiments.

REQUIREMENTS:

- 24-well culture flasks (Nunc, Denmark) – these are polystyrene flasks with a negatively charged ionised base, encouraging cell attachment.
- Culture medium: The preferred medium for breast epithelial cells was a combination of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 (Sigma), with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin.
- Sterile glass Pasteur pipettes
- 5, 10 and 25 ml plugged sterile pipettes with automatic pipette gun
- 15 and 30 ml sterile conical centrifuge tubes
- Waste beaker
- Labcoat and protective gloves with facemask
- Marker pen and adhesive tape

PROCEDURE:

The cell line was located and the index carefully checked prior to removing the ampoule from the liquid nitrogen store. The ampoule was defrosted in 3-5 minutes (by keeping it within a clasped hand) and opened under the LFC. The cells were dispersed (by gentle tapping) and the contents of the ampoule transferred to a 30 ml centrifuge tube using a 1ml pipette. Cryoprotectant (DMSO) was removed by centrifuging at 1200 rpm for 3minutes. The cells were resuspended in 1ml of culture medium and transferred to a culture flask containing 15 ml medium (DMEM/F12 with 10% FCS) equilibrated with 5% CO₂ gas and incubated at 37° C in 100% humidity.

3.4.2.3 SUBCULTURE METHOD

INTRODUCTION:

A primary culture, once subcultured (or *passaged*) is then known as a cell line. The MCF-7 cell line used in this study had already undergone 296 *passages*.

Monolayer cell cultures proliferate usually according to a set pattern. After seeding, a lag period is entered followed by an exponential or log growth phase. When the surface area of the culture flask or the available substrate is occupied, the cell concentration reaches confluence. Subculture should take place just before, or at the time it happens, as cells will begin withdrawing from the cell cycle and take longer to recover when reseeded. MCF-7 cells have a tendency to clump, so passaging prior to confluence was essential.

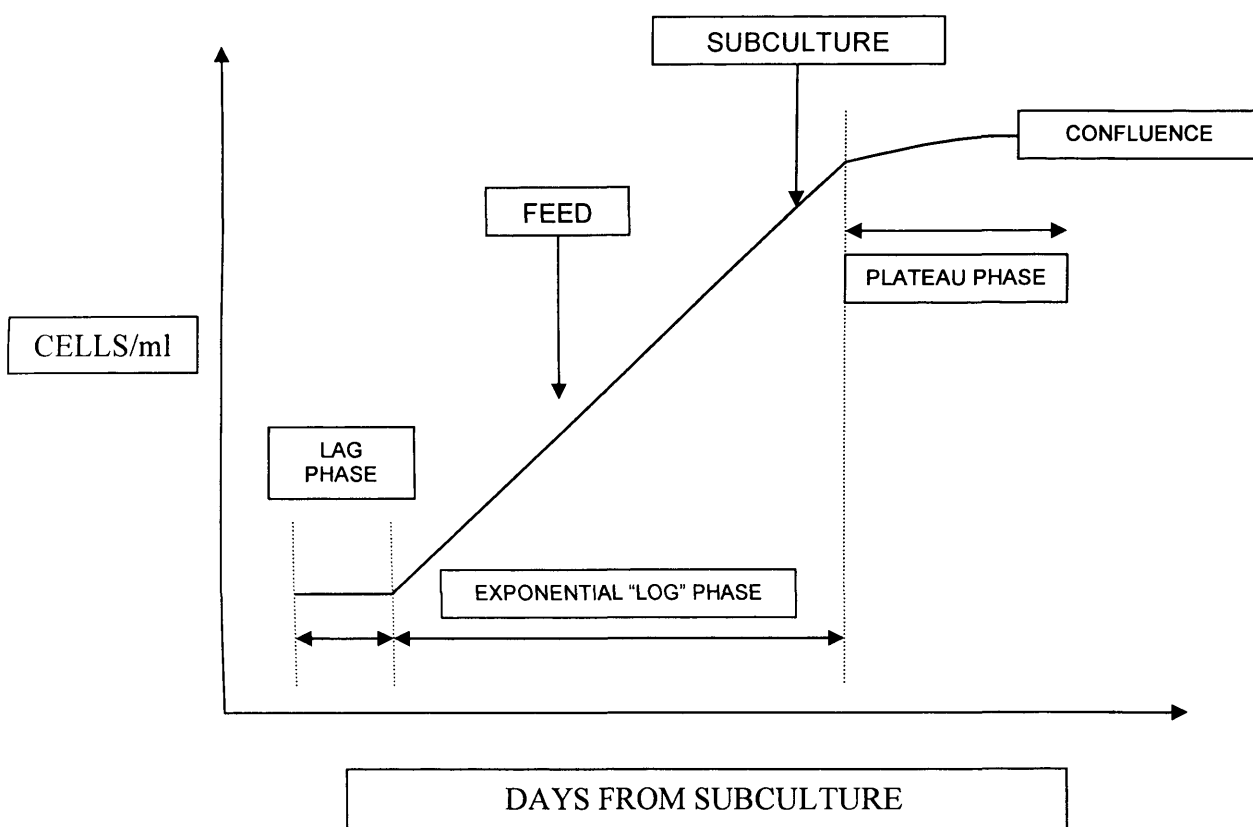


FIGURE 3.4.1 Standard logarithmic cell growth curve.

Standard cell growth curve (semi log plot) showing the different phases of growth and the timings for feeding and subculturing respectively.

PROCEDURE:

ADDITIONAL REQUIREMENTS:

- 0.02 % EDTA (ethylenediaminetetraacetic acid) combined with PBS (Phosphate buffered saline) (Sigma). This is prepared by mixing 5ml of 2% EDTA (Sigma) in 500ml PBS giving 0.02% EDTA/PBS.
- Trypsin at a concentration of 0.5mg/ml (0.02%).

The culture flasks were removed from the incubator and examined under the microscope to ascertain cell density, contamination and the general health of the cells. Every time these cells were passaged, 2 flasks were prepared for transfer of the cells so that with every experiment, there was a back-up supply of MCF-7's available to harvest. It was the policy of this lab to subculture once at least 80% confluence was obtained (assessed visually). Provided the cells were considered ready for subculture, the medium was discarded completely inside the LFC. The cells were washed three times using 10ml of 0.02% EDTA/PBS. The purpose of washing was to remove traces of serum that would otherwise inhibit the action of trypsin and deplete the divalent cations, which were necessary for cell adhesion. Cell detachment from the culture flask was achieved by adding 1ml of trypsin (0.02%) and incubating in a humidified environment for 3-5 minutes. Once detachment had occurred, the cells were dispersed and washed in 5ml medium (DMEM/F12 with 10% FCS) by centrifuging at 1500 rpm for 3 minutes. The cells were then resuspended in 8ml of medium. Seeding densities of 1:4 and 1:8 were prepared in two-subculture flasks by adding 2ml and 1ml respectively of this cell suspension to each. The flasks were then placed in a humidified incubator at 37° C and checked in an hour to ensure that no major pH change had occurred.

COUNTING THE CELLS:

Cell counts were performed after every subculture or administration of trypsin for the undertaking of experiments. This was achieved using a hemocytometer (Improved Neubauer). This device allows the cells to disperse within an optically flat chamber of known depth (or volume). When counted within a defined area under the microscope, cell concentration can be derived.

ADDITIONAL MATERIALS:

- Yellow pipettor tips
- Automatic pipette (up to 200 μL)
- Hemocytometer with coverslip

It was not the policy of this lab to routinely stain the cells with trypan blue as the MCF-7's, which were viable, were easily distinguishable from those which were not (these looked like "shadows"). The surface of the slide and coverslip were both cleaned with 70% alcohol prior to use. The edges of the coverslip were wetted slightly and then placed down over the grooves of the silver-lined counting area. Proper placement should have resulted in the appearance of "Newton's rings" (rainbow-like colours between the coverslip and the slide). The cells, after being trypsinised, were dispersed with the yellow-tipped pipette and a small amount withdrawn. This was then taken to the edge of the coverslip and a drop drawn from the edge of the coverslip by capillarity. The same was repeated on the other side. Any surplus fluid outside the coverslip was blotted away. The slide was then placed under the microscope and the central area of the grid, bounded by parallel lines was focused on. The number of viable cells (n) within this 1mm^2 was counted. The number of cells/ml (c) in this area was calculated by the formula $c = n \times 10^4$. The volume of the original sample then determines the total number of cells. For example, if the original suspension volume was 8ml, the total number of cells in the suspension would be $8 \times c$, or $8 \times n \times 10^4$. Determining the seeding density required for plating the cells will be discussed later.

FEEDING THE CELL CULTURE:

Incubated culture flasks were inspected at least once daily for contamination. Cells required feeding approximately every 2 – 3 days. The need for feeding was based on the appearance of the culture medium (changes in pH) and the morphology of the growing cells. To feed the culture, the medium was decanted under the LFC using the usual start-up procedure. Fresh medium (25ml) was replaced and equilibrated with 5% CO₂ using a Pasteur pipette. The culture flask was then replaced into the incubator and checked again in an hour to ensure that no major pH change had occurred.

FREEZING THE CELLS:

After the first passage was created from the thawed MCF-7's, the remaining cells from the thawed passage (p) 296 was frozen to create a back-up supply of cells for the experiment. The cryoprotectant used is DMSO (Lovelock & Bishop 1959), at a concentration of 10%.

PROCEDURE:

ADDITIONAL REAGENTS:

- L-15 medium (Leibovitz, Sigma) with 10%FCS and 1% penicillin/streptomycin.
This medium was normally used whenever cells need to be transferred, as it is not CO₂ dependent, and therefore maintains its pH in air.
- DMSO (10%) – this was made by adding 1ml of DMSO to 9ml of L-15 medium.
- 0.02 % EDTA/PBS
- 0.02 % trypsin
- Cryoflasks (2ml) (Sarstedt, Germany) were used to place cells for storage. Each flask was individually labelled with the details of the passage used e.g. MCF-7, p296 dated 12/4/05.

The procedure was initially the same as for subculture as previously described. Following trypsinisation and centrifugation, the cells were resuspended in 6ml of medium containing 10% DMSO. To each labelled flask (6 in total), 1ml of cells in medium were added. The cooling rate at which the cells were frozen needed to be slow at first to allow water to leave the cell but not so slowly as to allow ice crystal formation. This was achieved by placing the vials into the head of a freezing chamber (Taylor Wharton neck plug cooler) for 20 minutes before lowering the height of the ampoules quickly, which resulted in a super freeze at -70 to -90 °C. The cryoflasks were then transferred to the liquid nitrogen containers into carefully arranged rods, where the temperature dropped rapidly to between -180 and -190 °C. A careful record was made of all cells that were frozen or thawed.

PLATING THE CELLS:

The initial procedure was similar to that described for subculture (section 4.4.2.3 pg 145-147). Before centrifuging the cells, the cells were filtered using a $40\mu\text{m}$ nylon cell strainer. The purpose of this step was to remove clumps of cells. The filtered medium and substrate was then centrifuged at 1500rpm for 3 minutes. The medium was discarded and the cells resuspended in 5ml medium. After dispersal, the cell concentration was estimated using the hemocytometer as previously described at a plating density of 20000 cells/well.

3.4.3 ESTABLISHING THE DOSE RESPONSE

INTRODUCTION

Initial experiments were carried out to optimise culture conditions prior to experimentation with cytotoxic agents. The optimised culture conditions were to maintain the MCF-7 cells in DMEM/F12 supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. The cells were grown in 100% humidity after equilibrating in 5% CO₂ at 37 °C at a plating density of 20000cells/well.

The initial dose ranges chosen for this experiment were based on our collaborators' (Professor John Hartley, Department of Oncology) previous experience in establishing dose response in various cell lines including MCF-7 (Friedmann et al. 2004; Friedmann et al. 2006; Kupchinsky et al. 2004). Several experiments were then performed to optimise the dose range for each drug.

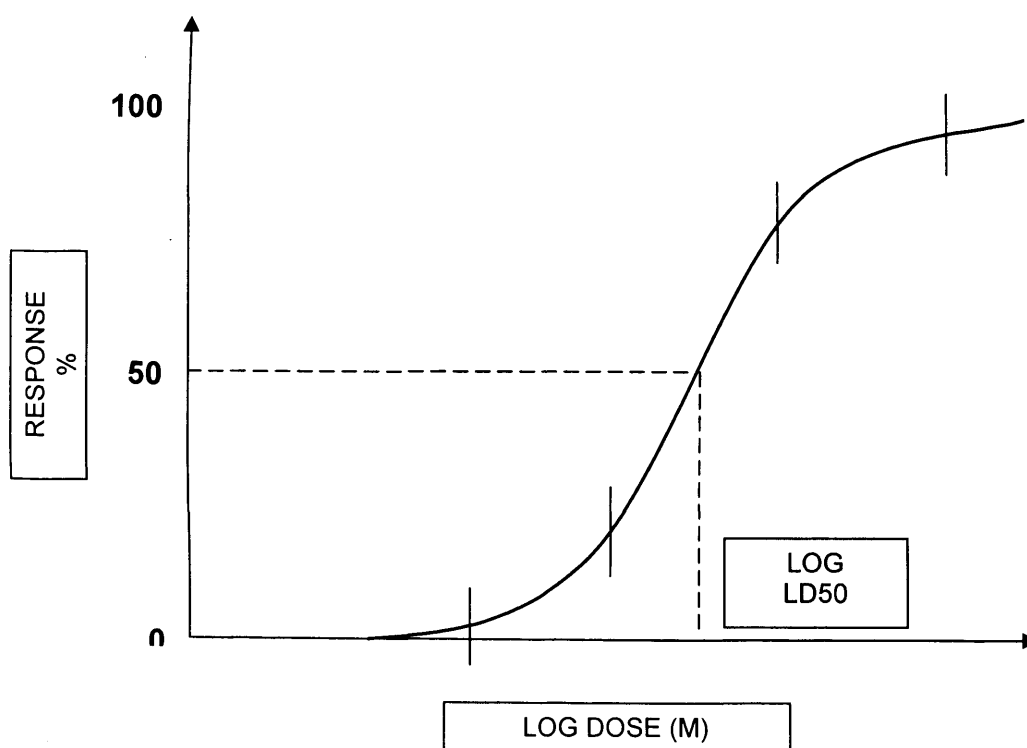


FIGURE 3.4.2 A typical sigmoidal dose response curve.

PROCEDURE:

ADDITIONAL REAGENTS/MATERIALS:

- Electronic scale (A&D instruments, Abingdon, Oxford, UK)
- Hanks' balanced salt solution (HBSS) (with 1% bovine serum albumin (BSA), gentamycin (1:1000) and amphoterecin (1:100))
- 0.2µm sterile filters
- 5 and 10 ml sterile syringes
- 100% ethanol
- 1M Hydrochloric acid (HCL)
- DMSO
- Water
- Whirly mixer
- 2ml and 5ml flat bottom containers with lids
- 1000µl, 200µL and 20µL graduated pipettes and pipette tips

DRUG	APPEARANCE	MOLECULAR WEIGHT(MW)	SOLUBILITY	RECOMMENDED STOCK SOLUTION	STORAGE REQUIREMENTS
DOXORUBICIN	RED POWDER	580.0	WATER	1mM	2-8° C
MELPHALAN	WHITE POWDER	305.2	100 % ETHANOL AND 1M HCL	10mM	ROOM TEMP IN DARK ENVIRONMENT
PACLITAXEL	WHITE POWDER	853.9	DMSO	1mM	2-8° C
CISPLATIN	COLOURLESS FLUID	3.3 mmol	WATER	3.3mM	ROOM TEMP IN DARK ENVIRONMENT

TABLE 3.4.1 Drug characteristics.

To ensure consistency, the top concentration for each drug was concentrated X 20.

Drugs were freshly diluted in culture medium and filter sterilised prior to administration.

Control cells received vehicle alone. All dose ranges (except paclitaxel) consisted of 10-fold dilutions from a top concentration initially. Drug dilutions were performed under the LFC using carefully labelled 5ml cylindrical flasks and graduated micropipettes.

The drugs were always added in the order of doxorubicin first, followed by melphalan, paclitaxel and cisplatin respectively. The time taken from drug dilution to application in all 4 plates was no longer than 30 minutes. After application of drug, the plates were carefully labelled and sealed with adhesive tape, then placed in a humidified incubator at 37° C. The effect of the drug was assessed under the microscope daily for up to 1 week. After several experiments, the dose ranges were optimised as shown below after a 72 hour exposure.

DRUG	TOP CONC	1 ST DIL	2 ND DIL	3 RD DIL	4 TH DIL
DOXORUBICIN (nM)	100	50	25	12.5	6.25
MELPHALAN (μM)	25	12.5	6.25	3.13	1.56
PACLITAXEL (nM)	50	25	12.5	6.25	3.13
CISPLATIN (μM)	25	12.5	6.25	3.13	1.56

TABLE 3.4.2 Drug dilutions.

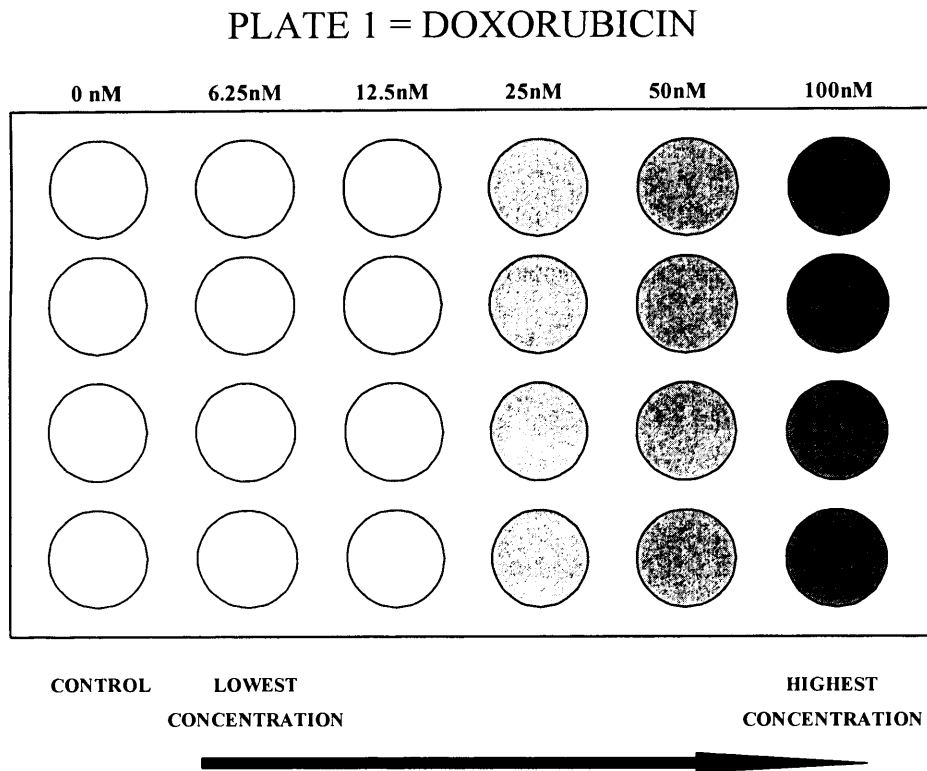


FIGURE 3.4.3 Standard plate arrangement.

Final appearance of plate arrangement after the dose response was validated (doxorubicin shown).

FIXING THE PLATES:

The purpose of this procedure was to create a permanent visual representation of how the dose response range for each drug was achieved.

REAGENTS:

- 4% Formalin
- Hematoxylin
- PBS (Phosphate buffered saline)

PROCEDURE:

The entire process was non-sterile and was therefore performed outside the LFC.

4% formalin was prepared by mixing 50ml of ~40% formalin solution (Sigma, UK) in 500ml PBS (Sigma, UK).

The plates to be fixed were removed and the medium from each well removed using a non-sterile pipette. The cells were then washed with PBS (1ml) after which formalin (1ml) was added to each well using a graduated pipette. The plates were covered, sealed and then placed in the refrigerator overnight.

The following day, the plates were removed and the formalin decanted into a running sink. The cells were then washed with PBS (1ml). Hematoxylin (1ml) was added to each well and the plates allowed to stand for at least one hour.

Following this, the hematoxylin was removed from each well (and stored for later use). The plates were then washed in running water twice. This resulted in a blue colour. After the third wash, the wells were filled with approximately 1ml of water each, and the plates sealed and stored in the refrigerator at 4-8°.

3.4.4 GRANULOSA CELL CULTURE

3.4.4.1 GRANULOSA CELL SOURCE

Granulosa cells were isolated from human follicular fluid (hFF), collected from patients having oocyte recovery after stimulation for IVF. Patients underwent pituitary down-regulation for three weeks using a gonadotrophin releasing hormone (GnRH)-analogue followed by 7-10 days of ovarian stimulation with high-purity follicle-stimulating hormone (FSH). Once there was a sufficient ovulatory response, patients were administered human chorionic gonadotrophin (HCG) for 36 hours after which any follicle > 10mm in diameter was aspirated under ultrasound guidance.

3.4.4.2 MATERIALS/REAGENTS

In addition to the standard apparatus used to culture the breast cell line MCF-7 as previously mentioned, several additional reagents were required for this culture.

- Laminar flow cabinet (LFC)
- Centrifuge
- Humidified incubator providing 5% CO₂ and 20% O₂.
- Inverted microscope
- Autoclave
- Hemocytometer (Improved Neubauer)
- Trypan blue
- 50ml sterile conical flasks (plastic)
- 5ml sterile flat bottom tubes
- Virkon ® and 70% ethanol
- Waste beaker with suction
- Sterile graduated pipettes 1ml, 5ml, 10ml, 25ml

- Sterile 20 μ L, 200 μ L and 1000 μ L pipettes
- Sterile plastic Pasteur pipettes
- Sterile glass beakers
- 60% percoll solution (Sigma): This is a non-toxic, sterile, isosmotic fluid, which provides a density gradient for the separation of cells. Initially 100% percoll is prepared by adding 9ml to 1ml of 1.5M sterile sodium chloride solution (NaCl). 60% percoll is then obtained by mixing 15ml of 100% percoll with 10ml of HBSS.
- HBSS supplemented with penicillin/streptomycin (1%); amphoterecin 1:100 and gentamycin 1:1000
- Hyaluronidase (Sigma): This was prepared as a stock solution (10mg/ml) by reconstituting the vial with 3ml distilled water. The stock was divided into 500 μ L aliquots and frozen at -20°C for future use. A working concentration of 300 μ g/ml was achieved by diluting 450 μ L of stock solution with 15ml HBSS (33-fold dilution).
- DMEM/F12 medium (GIBCO) supplemented with antibiotics
- 20% FCS (GIBCO).

3.4.4.3 GRANULOSA CELL CULTURE PROCEDURE

A meticulous aseptic technique was essential. All experiments were carried out strictly under sterile conditions in a laminar flow cabinet. Human follicular fluid (HFF) was pooled from each patient and centrifuged at 1100 rpm for 10 minutes. The supernatant was discarded and the cells were washed with Hanks balanced salt solution (HBSS; Gibco Ltd.) containing amphoterecin (1:100) and gentamycin (1:1000). The granulosa cells were separated from red blood cells by centrifugation at 1100rpm on a 60% Percoll layer for 30 minutes. After Percoll separation the granulosa cells were washed with HBSS and the cell aggregates incubated with 2ml of HBSS containing 300µg/ml bovine testicular hyaluronidase (Sigma) for 15 minutes (to reduce clumping). After washing with HBSS the cell suspension was mechanically dispersed using sterile Pasteur pipettes to obtain maximal dispersion. A final wash with HBSS was performed and the resulting pellet resuspended with 2ml of culture medium consisting of DMEM/F12 (Gibco) containing 1% penicillin/streptomycin, amphoterecin (1:100), gentamycin (1:1000) and 20% fetal calf serum (FCS).

Cells were counted using the trypan blue exclusion method on a hemocytometer. This was performed by placing 50µl of medium containing cells in a petridish and adding an equivalent volume of trypan blue (0.4%). The cells were layered on a hemocytometer using the method described previously (section 4.4.2.3). Non-viable cells appeared deep blue in colour due to membrane permeability while viable cells excluded the dye.

Cells were plated at a density of 20000 cells/well in 24-well non-treated culture plates (Nunc) and incubated in a water-saturated incubator with 5% CO₂ at 37°C throughout the experimental period. Non-treated plates were chosen as the viability of the granulosa cells was improved following separation from the medium compared to the coated plates employed for the MCF-7's.

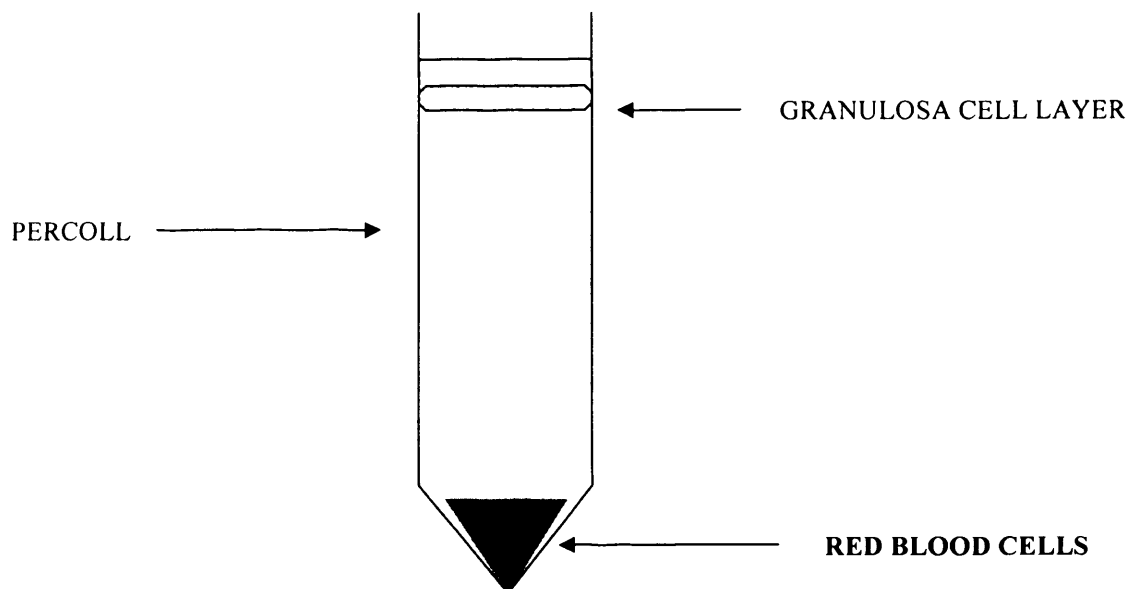


FIGURE 3.4.1 Percoll separation of granulosa cells.
Diagram showing percoll separation of GC's following centrifugation.

DRUG ADMINISTRATION:

Drugs were added in an analogous fashion to that used for the breast cells and at the same concentrations. After initial experiments, optimal exposure for the granulosa cells was 48 hours.

3.4.5 FLOW CYTOMETRY

3.4.5.1 INTRODUCTION

These experiments took place in the Department of Haematology, which was in close proximity to the cell culture laboratory. Mr Arnold Pizzey performed the flow cytometry after the samples were adequately prepared. A commercial apoptosis detection kit, Annexin-V-Fluos (Roche) was used for all experiments.

3.4.5.2 GENERAL REQUIREMENTS

- Mini-centrifuge
- Gilson pipettes – 20, 200 and 1000 μ L
- Plastic Pasteur pipettes (non-sterile)
- 2ml Eppendorf tubes
- 2ml flat bottom tubes with covers
- Cytometer tubes
- Marker pen
- Phosphate buffered saline (PBS)
- 0.02% EDTA
- 0.02% trypsin
- L-15 medium

FOR STAINING:

- Annexin-V-Fluos labelling reagent (250 μ L, stored at -20°C, Roche Diagnostics Germany)
- Annexin V buffer (made in-house): To make 500ml of this solution, 1.19 g Hepes was dissolved in 500ml-distilled water. 100ml of 6M NaOH was prepared by adding 24g of NaOH to 100ml-distilled water. Using the pH meter, this solution was slowly added to the Hepes solution until a pH of 7.4 was achieved. To this solution was added 140mM NaCl (4.09g) and 5mM CaCl₂ and placed on the magnetic stirrer. Once made this buffer can be stored at 2-8°C for up to 3 months.
- Propidium Iodide (PI, Invitrogen): This was usually stocked at 2mg/ml. A functional concentration of 50 μ g/ml was obtained by performing a 40-fold dilution with PBS.
- Annexin-V-Fluos labelling solution: This was made by prediluting 20 μ L Annexin-V-Fluos labelling reagent in 1ml Annexin-V buffer and adding 20 μ L PI.

3.4.5.3 FLOW CYTOMETRY PROCEDURE

At the end of the experimental period, cells were lifted from each plate in the same order that drug was administered. Culture medium was pooled from each well in quadruplicate, placed in eppendorf tubes and centrifuged at 13g for 1 minute. The supernatant was stored at -20°C for hormone assays. The resulting pellet contained dead cells, which were included in the flow cytometry analysis. Cells were then lifted from the plate using EDTA and 0.02% trypsin. Cells from the quadruplicate treatments were then pooled for flow cytometry. The cells were prepared for cytometry by first washing the cells with PBS and centrifuging at 13g for 1 minute. The cell pellet was then resuspended in 100 µl of Annexin-V-Fluos labelling solution. The cells were incubated for 10-15 minutes at room temperature in cytometer tubes before analysing in the flow cytometer. If a longer period was necessary, the cells were kept on ice in the dark for up to 1 hour until ready for analysis.

4. RESULTS IN VIVO STUDY

4.1 INTRODUCTION

The background, rationale and clinical study design have been discussed in chapters 2 and 3 respectively.

Briefly, pre-menopausal recipients of chemotherapy for breast cancer were analysed in two groups – a longitudinal arm (group 1) and a cross-sectional arm (group 2). In group 1, patients were offered ovarian reserve testing prior to receiving chemotherapy, and were then followed up longitudinally by performing further testing immediately following chemotherapy, and at several time-points afterwards (see chapter 3). In the cross-sectional study, patients were tested for ovarian reserve prior to chemotherapy (some patients were also part of the longitudinal study) and after chemotherapy when they had resumed regular menstrual cycles. Patients with oestrogen-sensitive cancer were recruited provided they had not taken tamoxifen or GnRH analogues (goserelin or leuprolin) for at least the preceding 12 weeks.

Controls were age-matched, had no medical illness, had previously proven fertility (defined by virtue of having childbirth) and had a normal menstrual history. Those on oral contraception were asked to discontinue it and use alternative contraception for at least 8 weeks prior to testing.

Unfortunately, the longitudinal arm of this study was severely affected due to the fact that most of the serum samples stored for analysis were lost in a freezer disaster over a Christmas holiday period. Samples which belonged to the controls and post-chemotherapy group were retrieved by repeating the tests where possible. This would explain most of the discrepancy in the number of samples (n) for the different hormones assayed. Other reasons for any discrepancy were as follows:

1. Some patients would not consent to the G test being performed, but allowed basal samples to be taken. As such stimulated and delta hormone levels for these patients could not be obtained.
2. After an analysis of preliminary data, it was decided not to continue testing of inhibin A, activin A, follistatin and Pro α C as these markers did not appear to be discriminatory.
3. With regard to biophysical parameters, it was not always possible to record certain variables such as ovarian volume or antral follicle count due to factors which impeded optimal viewing (such as distended loops of bowel in the pelvis).

4.2 TOLERABILITY

All tests performed were well tolerated, with no adverse effects reported.

4.3 STATISTICAL ANALYSES

A normality test was done to assess the distribution of data. Most variables in the cross-sectional data analysis had a gaussian distribution. As such, unpaired Student's t-tests were carried out to compare biochemical and biophysical parameters between patients and controls. One-way analysis of variance was used to compare baseline parameters only. A p value < 0.05 was considered statistically significant. When a significant difference was found, a post-hoc test (Bonferroni's multiple comparison) was used to compare differences between individual groups. Variables which displayed a non-gaussian distribution were compared using the Kruskal-Wallis test, with differences between individual variables analysed post-hoc using Dunn's multiple comparison tests.

Data in the longitudinal group, which had a normal distribution were analysed using the paired t-test. Non-parametric variables were analysed using the Wilcoxon signed rank test.

Unless specifically indicated, all parameters displayed a gaussian distribution and results displayed represent the mean value \pm the standard error of the mean (SEM). All Statistical analyses were performed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego California USA) and Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA).

4.4 CROSS-SECTIONAL DATA

4.4.1 CLINICAL PARAMETERS

4.4.1.1 CHRONOLOGICAL AGE

There was no statistically significant difference ($P > 0.05$) in age between all three groups. The mean age for patients in the pre-chemotherapy group was 35.2 ± 1.5 , for patients in the post-chemotherapy group 36.7 ± 0.6 and in the controls 34.5 ± 0.9 .

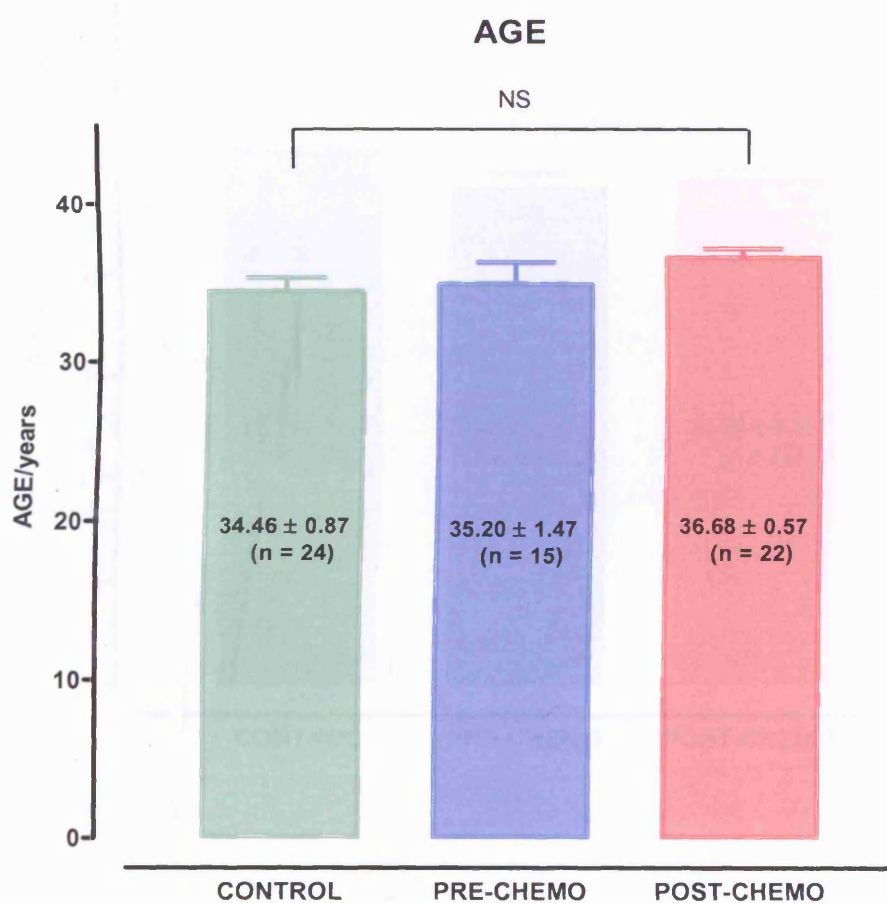


FIGURE 4.4.1.1 Mean age of study subjects.

Mean age (years) between all 3 groups. Not statistically significant ($p > 0.05$)

4.4.1.2 BODY MASS INDEX

There was no statistically significant difference in BMI (kg/m^2) between the 3 groups.

The mean BMI for patients in the pre-chemotherapy group was 24.27 ± 0.84 , for patients in the post-chemotherapy group 24.59 ± 0.53 and in the controls 26.04 ± 1.02 .

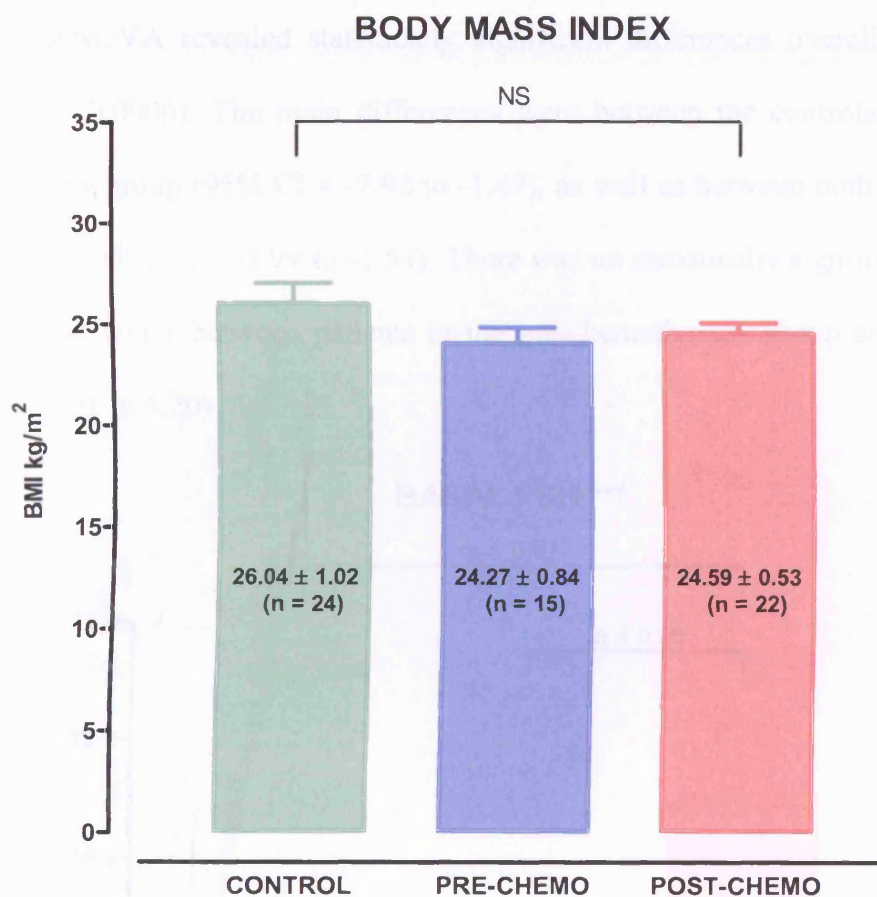


FIGURE 4.4 1.2 Mean BMI of study subjects.

BMI in all three groups – no statistically significant differences in the mean

4.4.2 BIOCHEMICAL PARAMETERS

All biochemical tests included basal estimates as well as stimulated and delta (stimulated – baseline, Δ) results following administration of the G-test.

4.4.2.1 FOLLICLE STIMULATING HORMONE

BASAL FSH:

One-way ANOVA revealed statistically significant differences overall among the 3 groups ($p = 0.0006$). The main differences were between the controls and the post-chemotherapy group (95% CI = -7.92 to -1.47), as well as between both patient groups (pre vs. post, 95% CI = -8.99 to -1.54). There was no statistically significant difference in basal FSH levels between patients in the pre-chemotherapy group and the controls (95% CI -3.01 to 4.20).

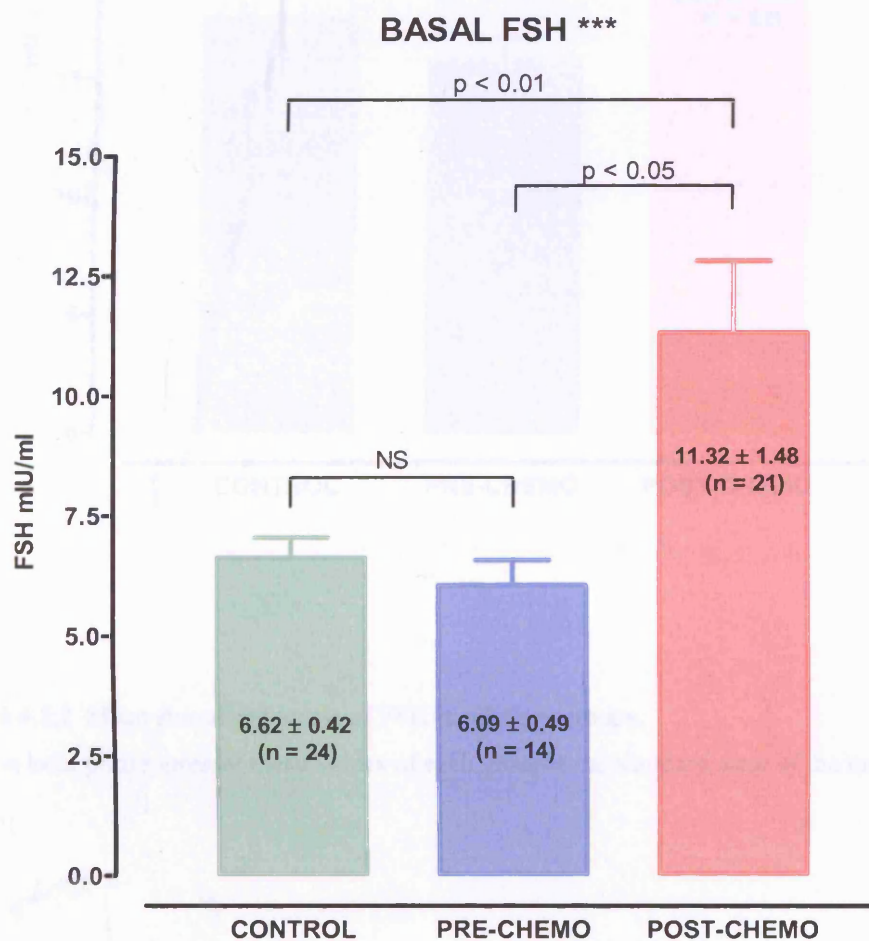


FIGURE 4.4.2.1 Basal FSH levels in all 3 groups.

Basal FSH levels between all 3 groups showed a statistically significant difference. *** $p < 0.001$

STIMULATED FSH:

One-way ANOVA revealed statistically significant differences among the 3 groups ($p = 0.005$). The main differences were between the controls and the post-chemotherapy group (95% CI = -10.89 to -0.91), as well as between both patient groups (pre vs. post, 95% CI = -14.36 to -1.13).

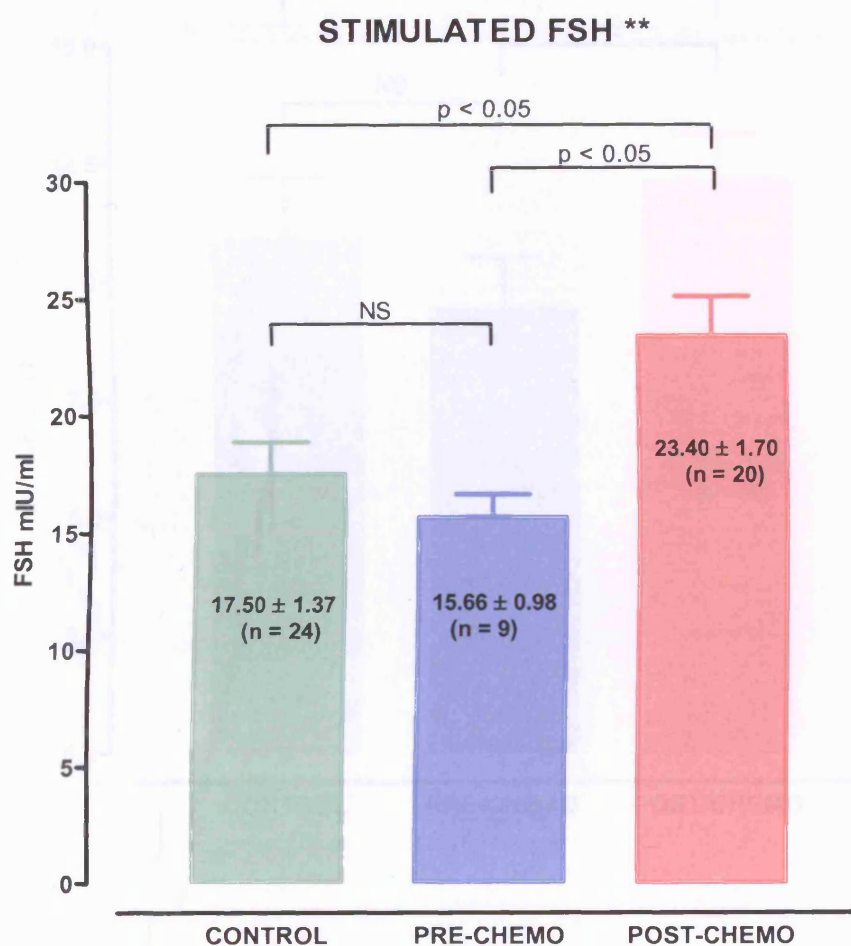


FIGURE 4.4.2.2 Mean stimulated levels of FSH in all three groups.

Numbers in bold print represent mean values of each group + the standard error of the mean (SEM).

** $p < 0.01$

DELTA FSH:

One-way ANOVA did *not* reveal any statistically significant differences among the three groups ($p = 0.42$).

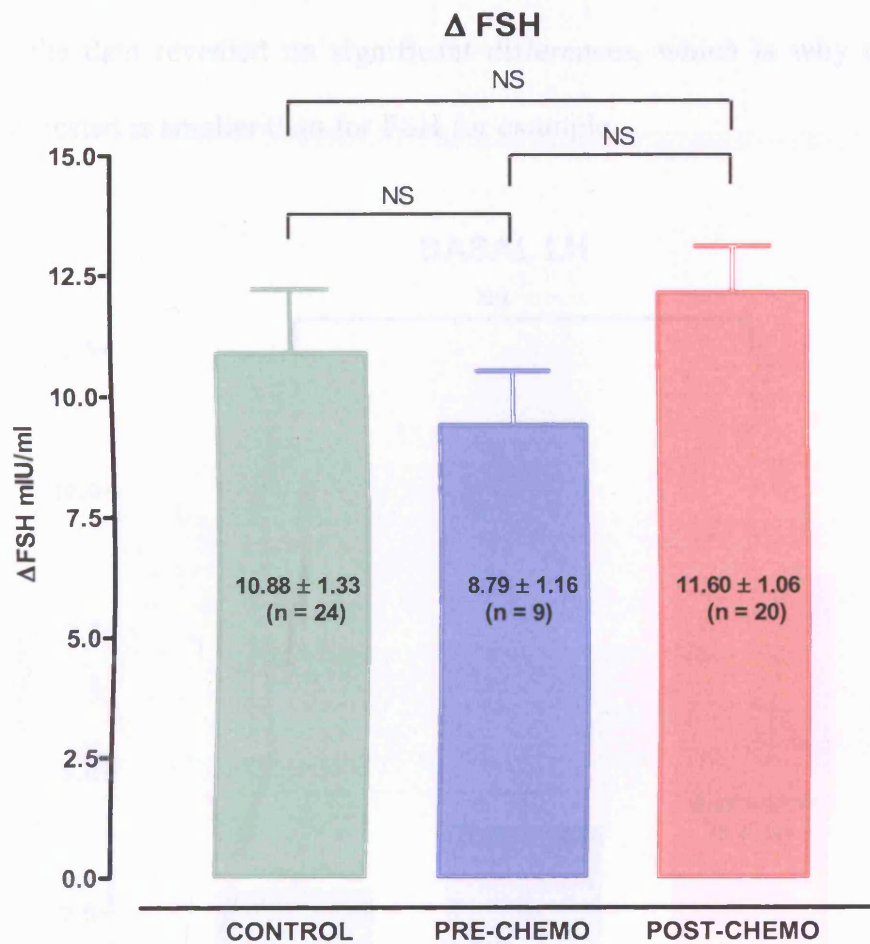


FIGURE 4.4.2.3 Delta (stimulated – baseline) FSH levels between all groups.

Not statistically significant ($p = 0.42$).

4.4.2.2 LUTEINISING HORMONE

There were no statistically significant differences in the mean values of basal LH, stimulated LH and delta LH between the controls, pre-chemotherapy and post-chemotherapy patients respectively. Further testing of LH was stopped after initial review of the data revealed no significant differences, which is why the number of samples (n) tested is smaller than for FSH for example.

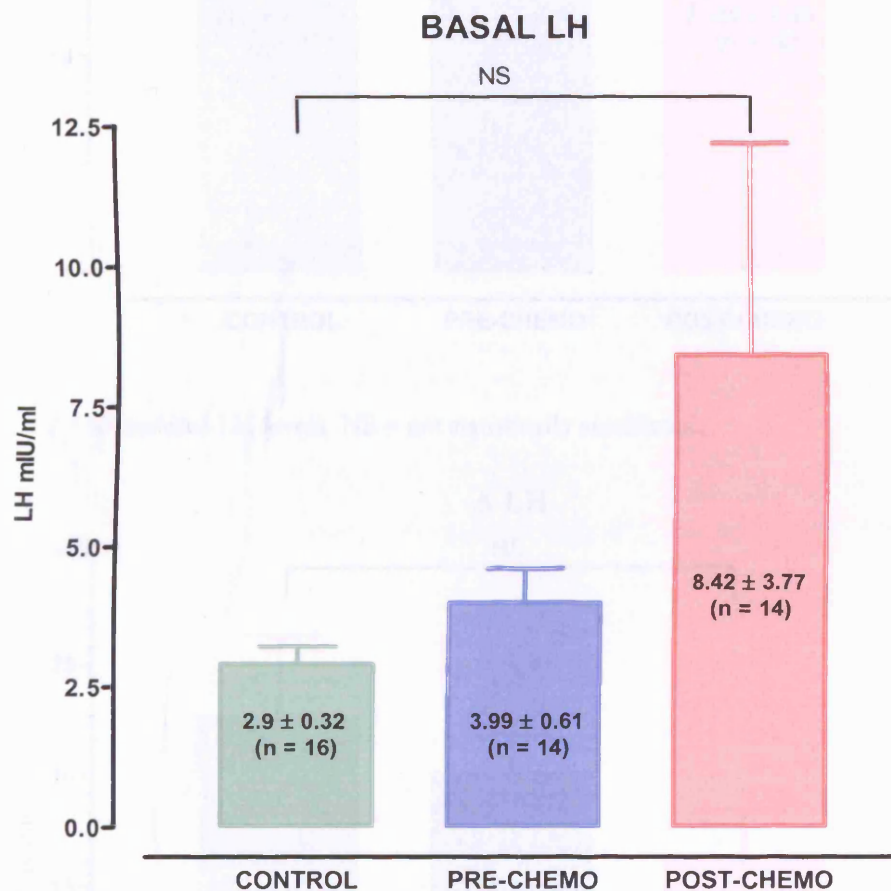


FIGURE 4.4.2.4 Basal LH in all 3 groups.

Mean basal LH values not significantly different between all three groups.

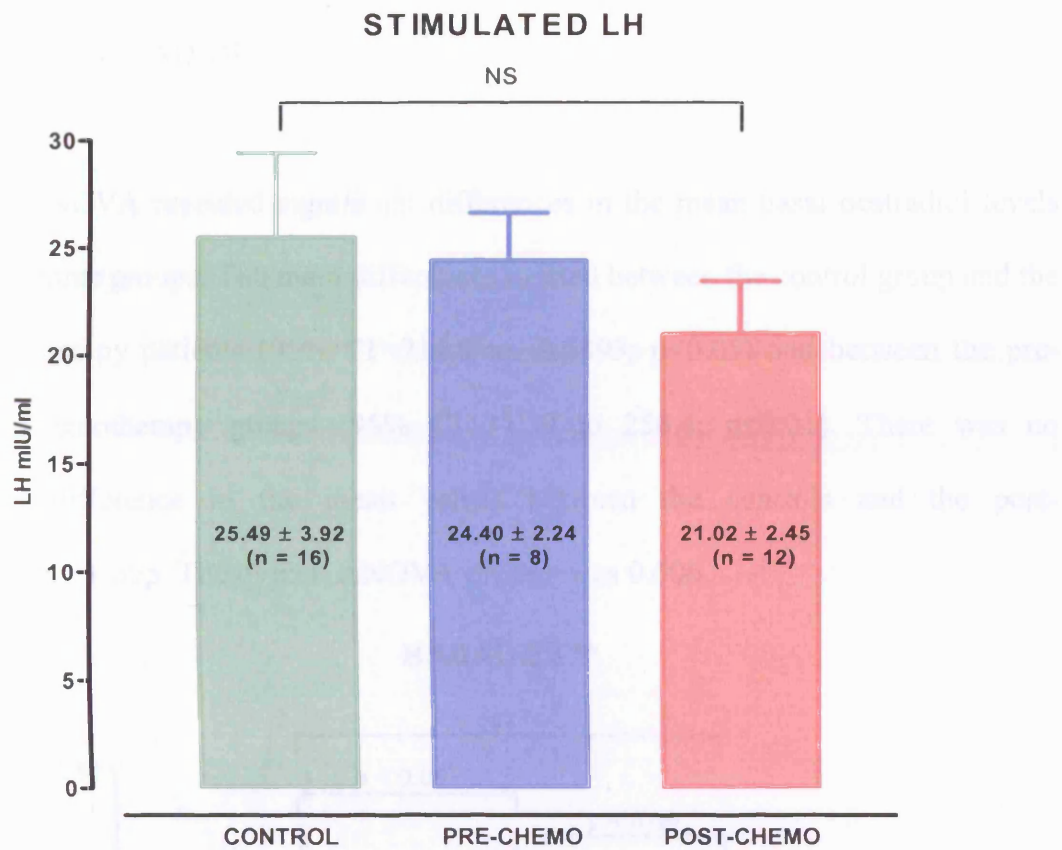


FIGURE 4.4.2.5 Stimulated LH levels. NS = not statistically significant.

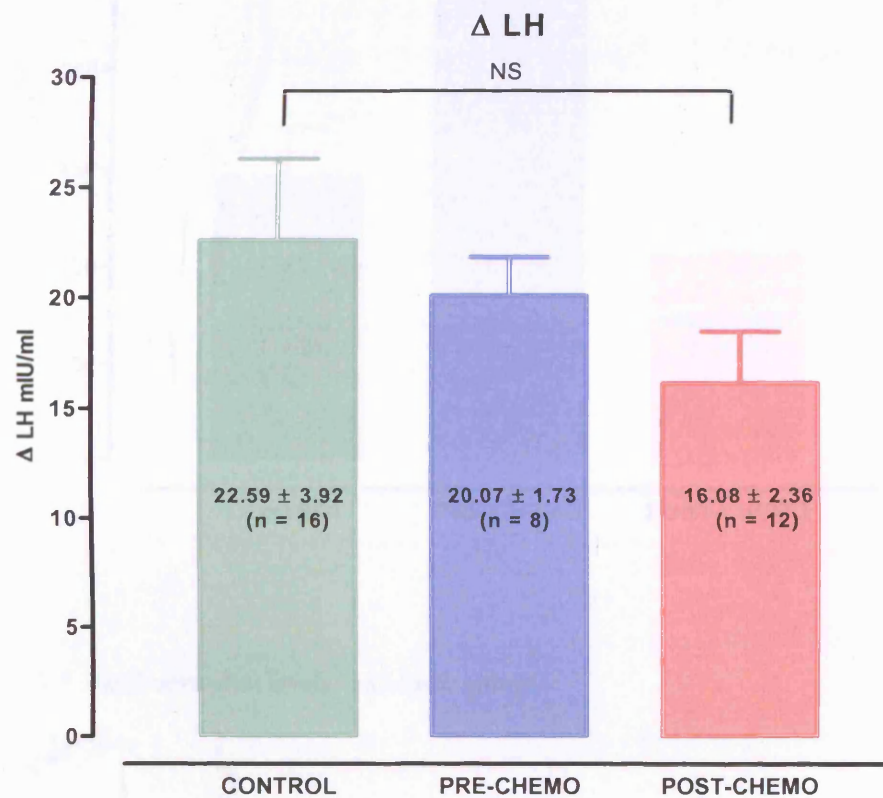


FIGURE 4.4.2.6 Δ LH levels.
NS = not statistically significant.

4.4.2.3 OESTRADIOL

BASAL E2:

One-way ANOVA revealed significant differences in the mean basal oestradiol levels among the three groups. The main differences existed between the control group and the pre-chemotherapy patients (95% CI -214.0 to -0.5893; $p < 0.05$) and between the pre- and post-chemotherapy groups (95% CI 37.39 to 256.4; $p < 0.01$). There was no significant difference in the mean values between the controls and the post-chemotherapy group. The overall ANOVA p value was 0.006.

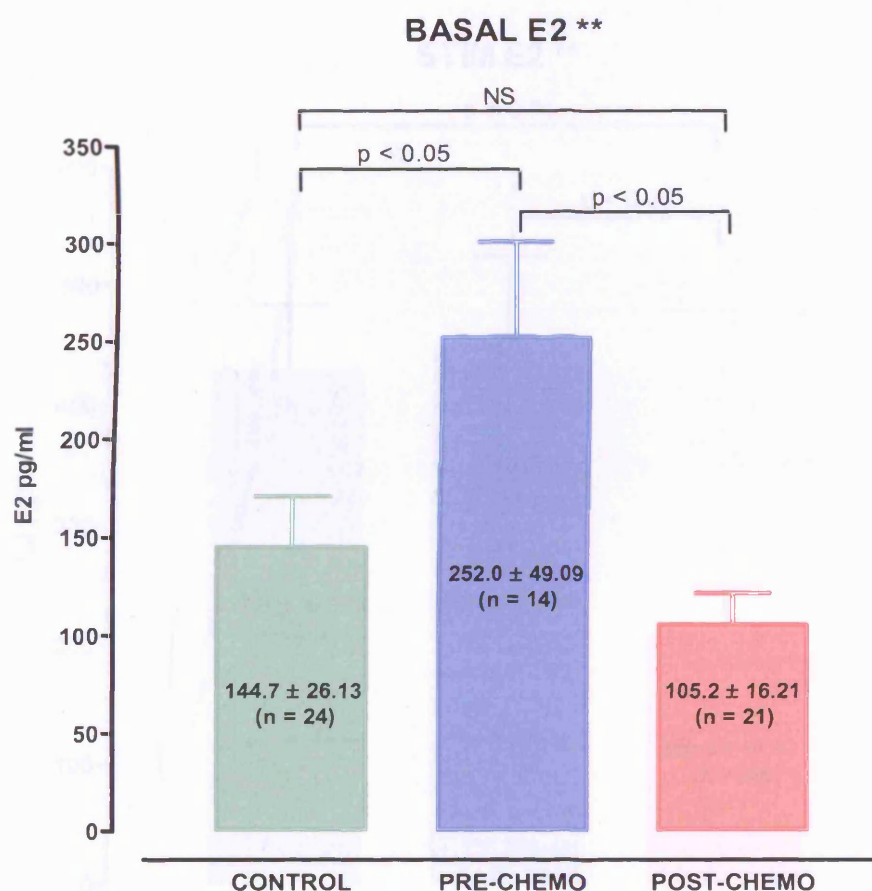


FIGURE 4.4.2.7 Basal oestradiol levels in all three groups.

** $p < 0.01$

STIMULATED E2:

ANOVA revealed significant differences between the mean stimulated oestradiol levels of the controls and the pre- and post-chemotherapy groups (overall $p = 0.005$) respectively. The overall difference was mainly accounted for by significant differences between the controls and the post-chemotherapy group (95% CI 45.05 to 391.0; $p < 0.01$), and between both patient groups (95% CI 10.67 to 469.3; $p < 0.05$). This was in contrast to the basal oestradiol ANOVA, where one of the main differences existed between the controls and the *pre-* chemotherapy group.

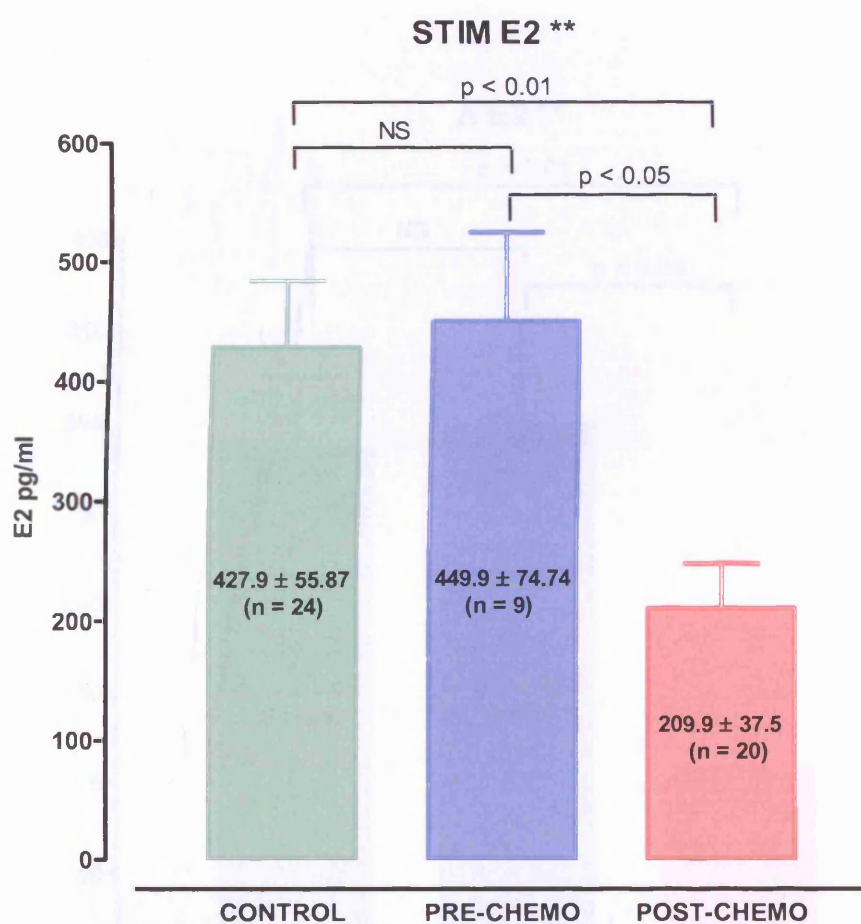


FIGURE 4.4. 2.8 STIMULATED E2 in all three groups.

The discrepancy between n for basal vs. stimulated oestradiol was either due to the fact that some patients defaulted from having the g-test performed, or from lost samples.

** $p < 0.01$

DELTA E2:

The means for the controls and patients were significantly different ($p = 0.002$). The main differences arose from comparisons between the controls with the post-chemotherapy group (95% CI 51.60 to 299.2; $p < 0.01$) and between the pre- and post-chemotherapy groups (95% CI 25.20 to 353.4; $p < 0.05$). This was unlike the case seen with basal E2, where there was no significant difference between patients in the post-chemotherapy group and the controls. This implies that delta E2 has discriminatory capacity as a marker of ovarian reserve (see discussion section 5.10).

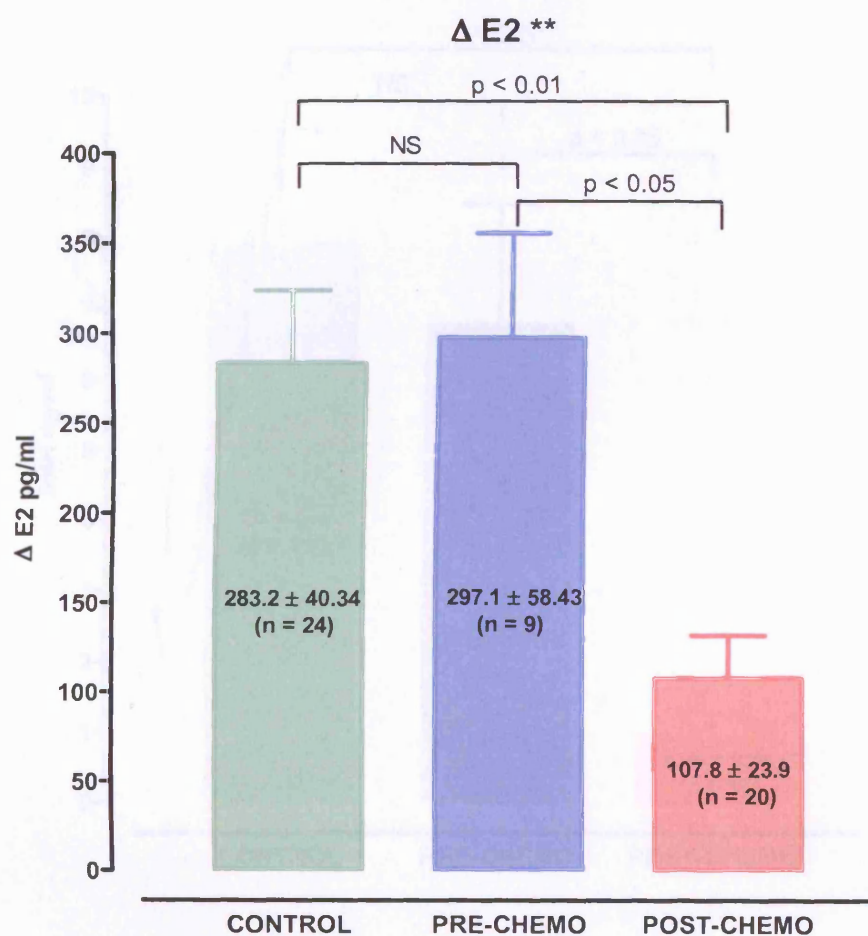


FIGURE 4.4. 2.9 Delta oestradiol in all 3 groups.

Delta oestradiol significantly lower in the post-chemotherapy group. ** $p < 0.01$

4.4.2.4 ANTI MÜLLERIAN HORMONE

BASAL AMH:

One-way ANOVA revealed highly significant differences ($p = 0.0006$) in mean basal AMH levels among the three groups. The greatest difference was seen between the controls and patients in the post-chemotherapy group (95% CI 2.68 to 11.21; $p < 0.001$); however a significant difference was also detectable between the pre-chemotherapy and post-chemotherapy patients (95% CI 0.62 to 11.02; $p < 0.05$). There was no significant difference between patients in the pre-chemotherapy group and the controls.

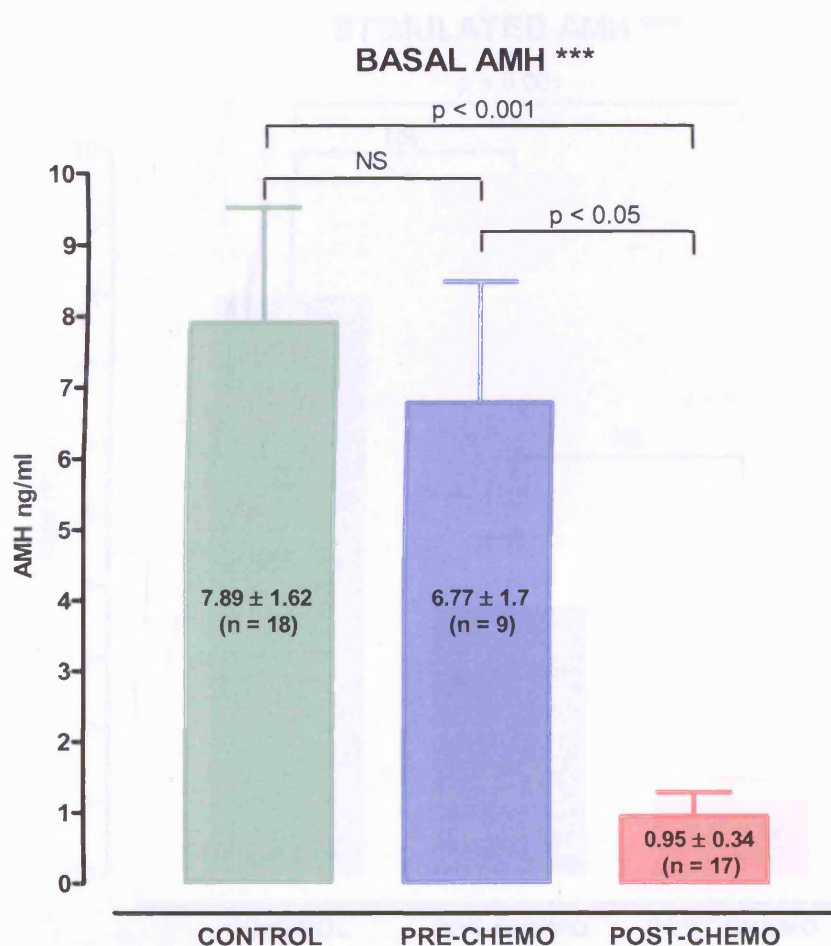


FIGURE 4.4.2.10 Mean basal AMH levels in patients and controls.

*** $p < 0.001$

STIMULATED AMH:

The mean values for stimulated AMH levels between patients and the controls were found to be significantly different ($p < 0.0006$). Most of the difference was found in the comparison between the controls and the post-chemotherapy group (95% CI 2.86 to 11.12; $p < 0.001$), although this was a gaussian approximation (Mann-Whitney test; this was likely due to the small sample size for the pre-chemotherapy group as a result of lost samples). As expected, stimulated AMH levels were not significantly different from basal AMH levels.

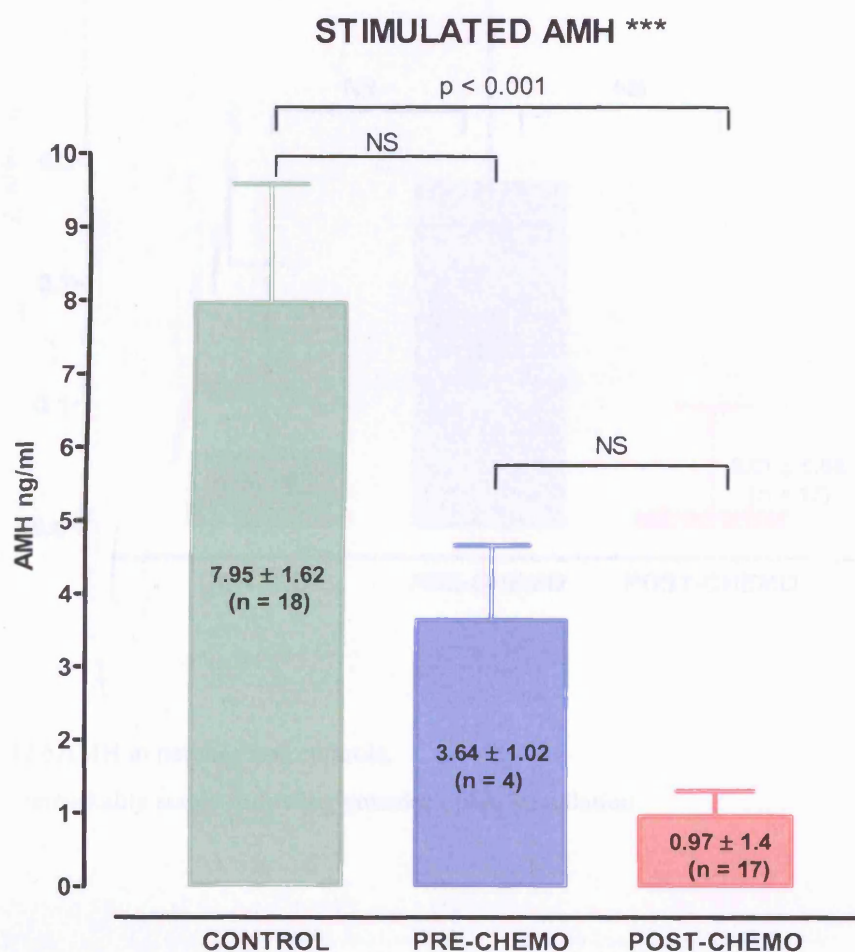


FIGURE 4.4.2.11 Stimulated AMH levels.

The distribution is non-gaussian for the post-chemotherapy group. *** $p < 0.001$.

DELTA AMH:

The mean Δ AMH levels between patients and the controls were not significantly different. This was likely due to the small number of samples obtained in the pre-chemotherapy group (due to lost/spoiled samples as described earlier).

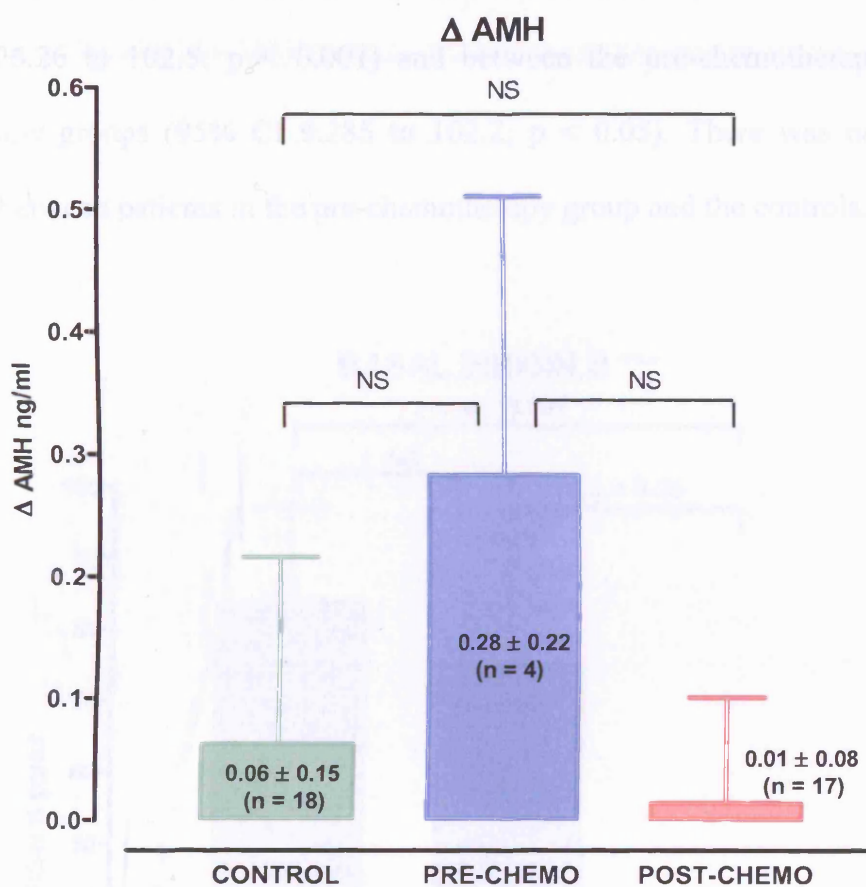


FIGURE 4.4.2.12 Δ AMH in patients and controls.

AMH levels are remarkably stable following gonadotrophin stimulation.

4.4.2.5 INHIBIN B

BASAL INHIBIN B:

One-way ANOVA revealed highly significant differences in the mean basal inhibin B levels among the three groups ($p = 0.0003$). Further analysis revealed the main differences in the means existed between the controls and the post-chemotherapy group (95% CI 26.26 to 102.5; $p < 0.001$) and between the pre-chemotherapy and post-chemotherapy groups (95% CI 9.285 to 102.2; $p < 0.05$). There was no significant difference between patients in the pre-chemotherapy group and the controls.

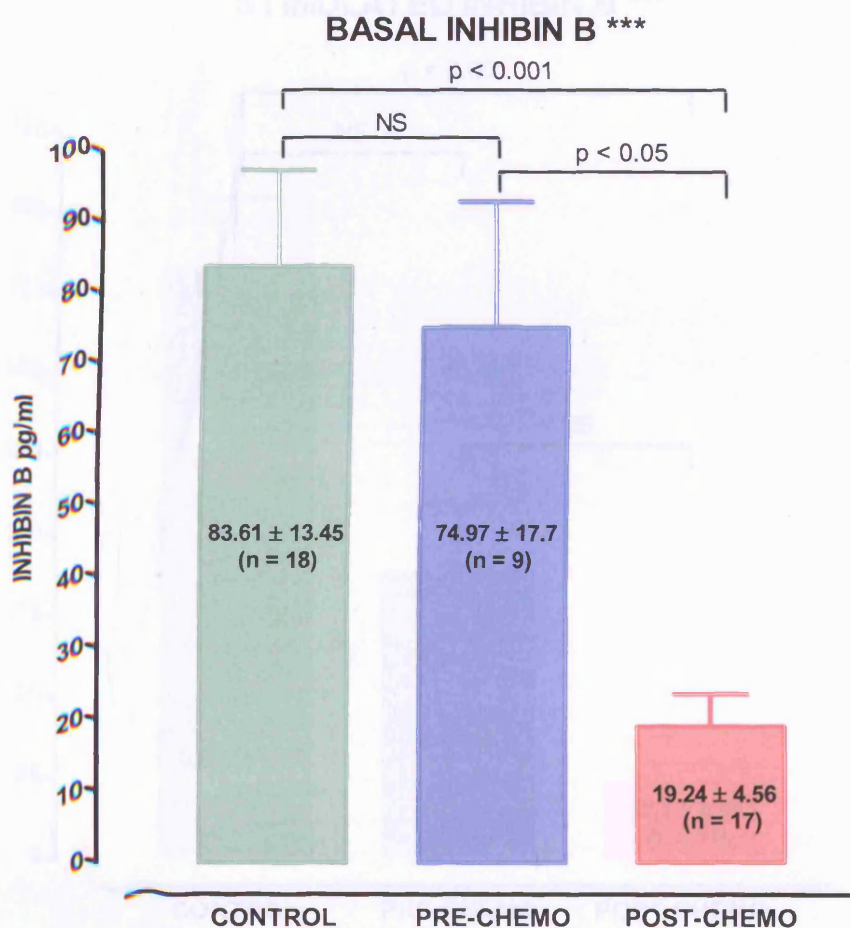


FIGURE 4.4.2.13 Mean basal inhibin B.

The distribution for the post-chemotherapy group was non-gaussian.

*** $p < 0.001$

STIMULATED INHIBIN B:

One-way ANOVA revealed highly significant differences between the mean levels of stimulated inhibin B among the three groups ($p < 0.0001$). The main difference existed between the controls and the post-chemotherapy group (95% CI 98.87 to 217.5; $p < 0.001$). The distribution for this group however was non-gaussian (Mann-Whitney test), and there was no significant difference in mean stimulated inhibin B between the controls and the pre-chemotherapy group (but the sample number is small ($N = 4$), as discussed previously for AMH).

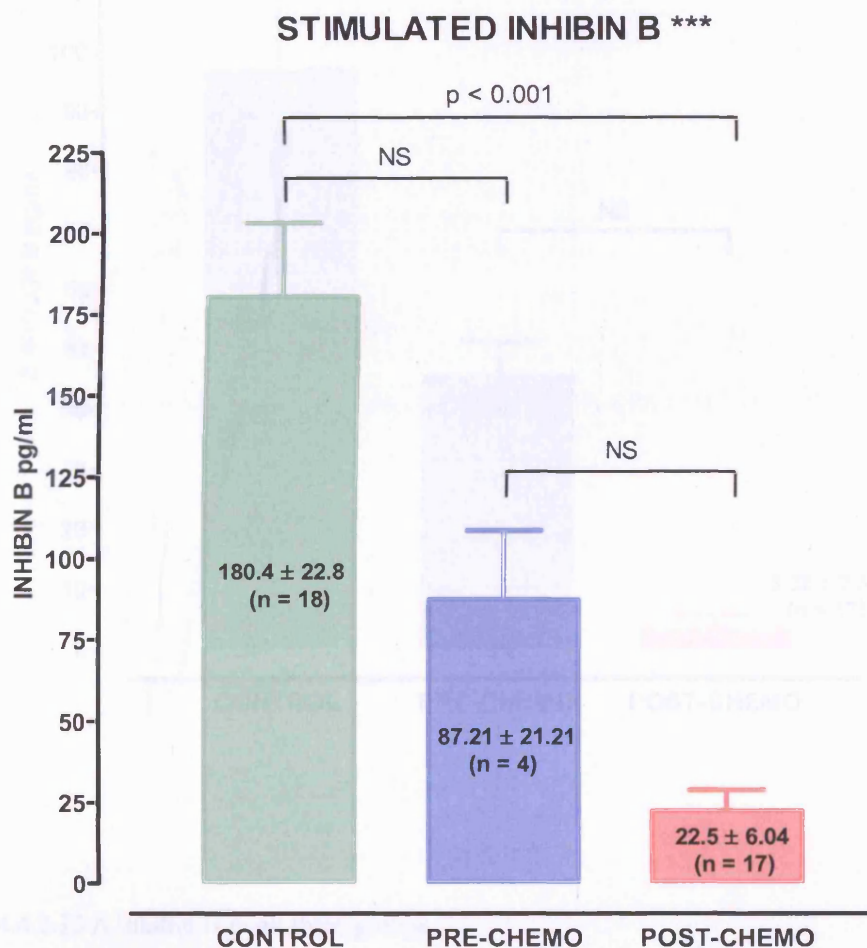


FIGURE 4.4.2.14 Stimulated inhibin B levels in all three groups.

*** $p < 0.001$

DELTA INHIBIN B:

There were highly significant differences in the mean Δ Inhibin B levels across the groups ($p < 0.0001$). This was again exemplified mostly by the difference in the mean between the controls and the post-chemotherapy group (95% CI 52.76 to 134.8; $p < 0.001$).

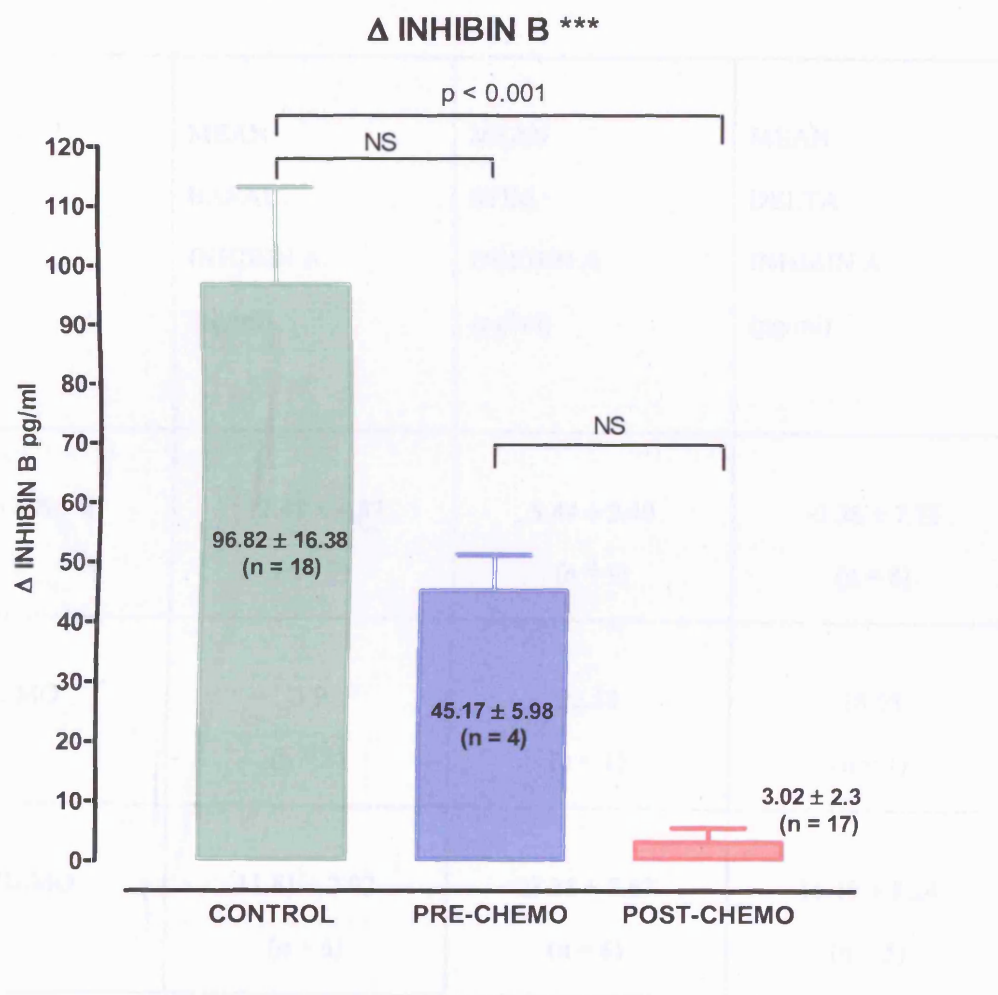


FIGURE 4.4.2.15 Δ Inhibin B in all three groups.

The distribution in patients is non-gaussian.

*** $p < 0.001$

INHIBIN A

There were no statistically significant differences in basal, stimulated or delta inhibin A levels among the three groups. Analysis of variance was calculated for basal samples only ($p = 0.53$), as the number of stimulated inhibin A samples in the patient groups were too small.

GROUP	MEAN BASAL INHIBIN A (pg/ml)	MEAN STIM INHIBIN A (pg/ml)	MEAN DELTA INHIBIN A (pg/ml)
CONTROLS	12.48 ± 4.87 (n = 9)	9.44 ± 2.49 (n = 9)	-3.36 ± 7.15 (n = 8)
PRE-CHEMO	3.9 (n = 3)	22.58 (n = 1)	18.68 (n = 1)
POST-CHEMO	11.81 ± 2.92 (n = 6)	23.38 ± 8.67 (n = 6)	15.19 ± 8.24 (n = 5)

TABLE 4.4.1 Inhibin A mean values in all three groups.

Sufficient data for analysis was obtained for the controls and the post-chemotherapy group only.

4.4.2.7 ACTIVIN A

One-way ANOVA revealed that the mean basal levels of activin A were not significantly different between patients and the controls ($p = 0.44$). Stimulated samples were low ($N = 2$) in the pre-chemotherapy group, making a one-way ANOVA unsuitable. However unpaired t-tests between the controls and the post-chemotherapy group revealed no differences in the means of stimulated and delta levels of activin respectively.

GROUP	MEAN BASAL ACTIVIN A (pg/ml)	MEAN STIM ACTIVIN A (pg/ml)	MEAN DELTA ACTIVIN A (pg/ml)
CONTROLS	367.2 ± 64.58 (n = 16)	338.6 ± 58.29 (n = 15)	-12.2 ± 67.1 (n = 15)
PRE-CHEMO	466.7 ± 85.51 (n = 8)	344.6 (n = 2)	-85.61 (n = 2)
POST-CHEMO	299.2 ± 95.10 (n = 7)	211.8 ± 44.69 (n = 7)	-34.5 ± 85.4 (n = 7)

TABLE 4.4.2 Mean activin A levels in patients and the controls.

4.4.2.8 FOLLISTATIN

One-way ANOVA was applied to basal follistatin results only as the sample size for stimulated levels in the pre-chemotherapy group was very small (N = 1). There were no significant differences in the means among the three groups ($p = 0.40$).

GROUP	MEAN BASAL FOLLISTATIN (pg/ml)	MEAN STIM FOLLISTATIN (pg/ml)	MEAN DELTA FOLLISTATIN (pg/ml)
CONTROLS	510.4 ± 121.7 (n = 9)	511.3 ± 79.38 (n = 9)	0.9222 ± 94.08 (n = 9)
PRE-CHEMO	490.8 ± 128.0 (n = 8)	1135.0 (n = 1)	228.1 (n = 1)
POST-CHEMO	302.6 ± 44.82 (n = 6)	393.4 ± 115.8 (n = 6)	90.82 ± 119.7 (n = 6)

TABLE 4.4.3 Mean follistatin levels in all groups.

The distribution was Gaussian for basal samples.

4.4.2.9 ACTIVIN A/ FOLLISTATIN RATIO

The difference between the mean basal A/F ratios were not statistically significant ($p = 0.07$). There were no significant differences in the means for stimulated and delta a/f ratios in the post-chemotherapy group. Meaningful analysis in the pre-chemotherapy group was impossible due to the small number of stimulated activin and follistatin results.

GROUP	MEAN BASAL A/F RATIO	MEAN STIM A/F RATIO	MEAN DELTA A/F RATIO
CONTROLS	0.78 ± 0.32 (n = 9)	0.69 ± 0.17 (n = 9)	0.60 ± 0.30 (n = 8)
PRE-CHEMO	1.84 ± 0.59 (n = 8)	0.11 (n = 1)	0.52 (n = 1)
POST-CHEMO	0.77 ± 0.18 (n = 6)	0.64 ± 0.19 (n = 6)	0.78 ± 0.19 (n = 6)

TABLE 4.4.4 Mean A/F ratios among all three groups.

4.4.2.10 PRO αC

One-way ANOVA was only possible for basal pro αC levels in all three groups due to insufficient samples from stimulated patients in the pre-chemotherapy group. However, the differences in the mean basal levels among the three groups were not significant (p = 0.95).

GROUP	MEAN BASAL PRO αC (pg/ml)	MEAN STIM PRO αC (pg/ml)	MEAN DELTA PRO αC (pg/ml)
CONTROLS	193.7 ± 47.40 (n = 15)	268.6 ± 38.96 (n = 14)	70.29 ± 31.15 (n = 14)
PRE-CHEMO	212.9 ± 49.19 (n = 8)	331.8 (n = 1)	186.4 (n = 1)
POST-CHEMO	214.2 ± 64.74 (n = 6)	307.3 ± 71.86 (n = 6)	93.06 ± 14.16 (n = 6)

TABLE 4.4.5 Mean Pro αC values in all three groups.

4.4.3 BIOPHYSICAL PARAMETERS

4.4.3.1 INTRODUCTION

A single operator with expert – level experience and technical ability performed all ultrasound examinations (Miss Anita Patel). On certain occasions, when Ms Patel was unavailable, it was necessary to obtain the assistance of another expert sonographer (Dr Bindhu Parikh). Inter-observer variability was verified to be low by randomly scanning three study subjects twice (with permission) independently on the same day by both sonographers and comparing the results.

All scans were performed prior to administration of the G-test. In 3 cases, scans were repeated 24 hours following the G-test (with permission). There were no noticeable differences in the biophysical parameters obtained prior to stimulation in the same study subjects (data not shown). Where paired readings (both ovaries) were not obtainable, the results were omitted from the analysis. All biophysical parameters, with the exception of Doppler indices passed normality testing (distribution was gaussian).

4.4.3.2 OVARIAN VOLUME

There were no statistically significant differences in total (TOV) and mean (MOV) ovarian volume among the three groups as assessed by one-way ANOVA (TOV: $p = 0.1$; MOV: $p = 0.1$).

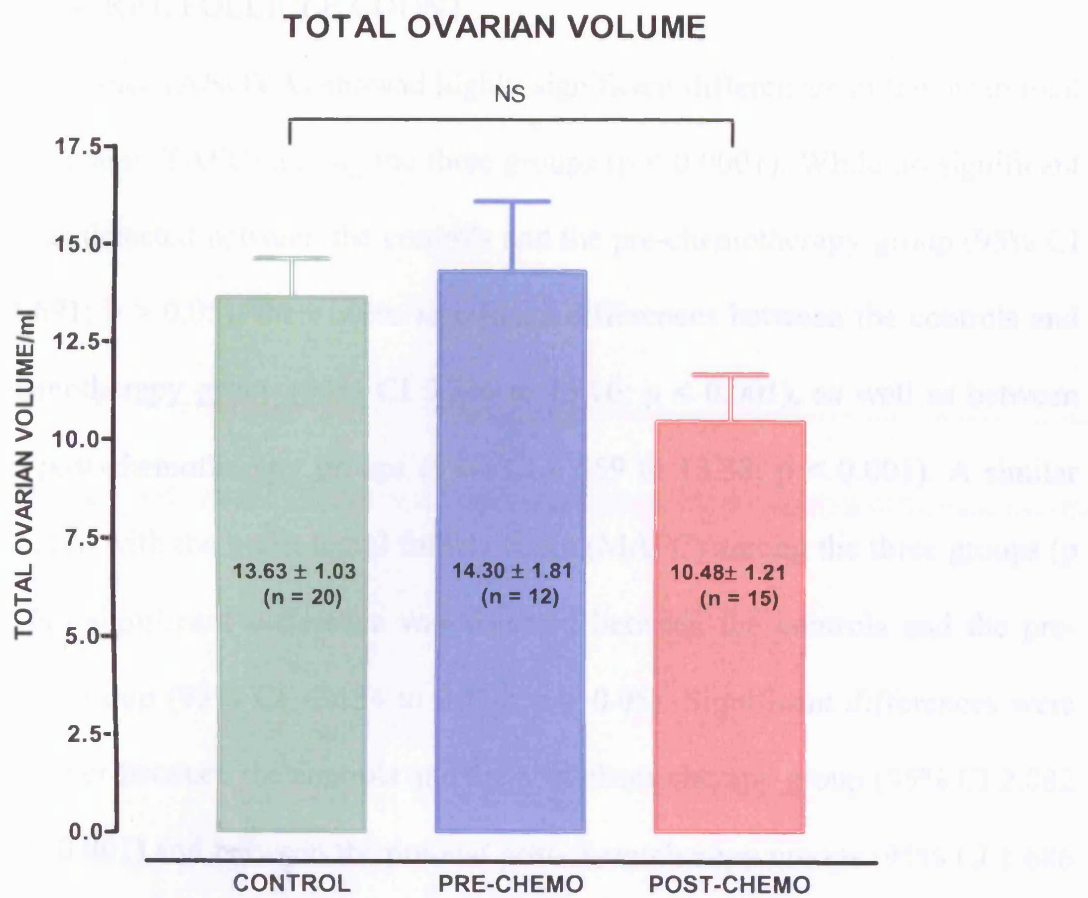


FIGURE 4.4.3.1 TOV between patients and the controls.

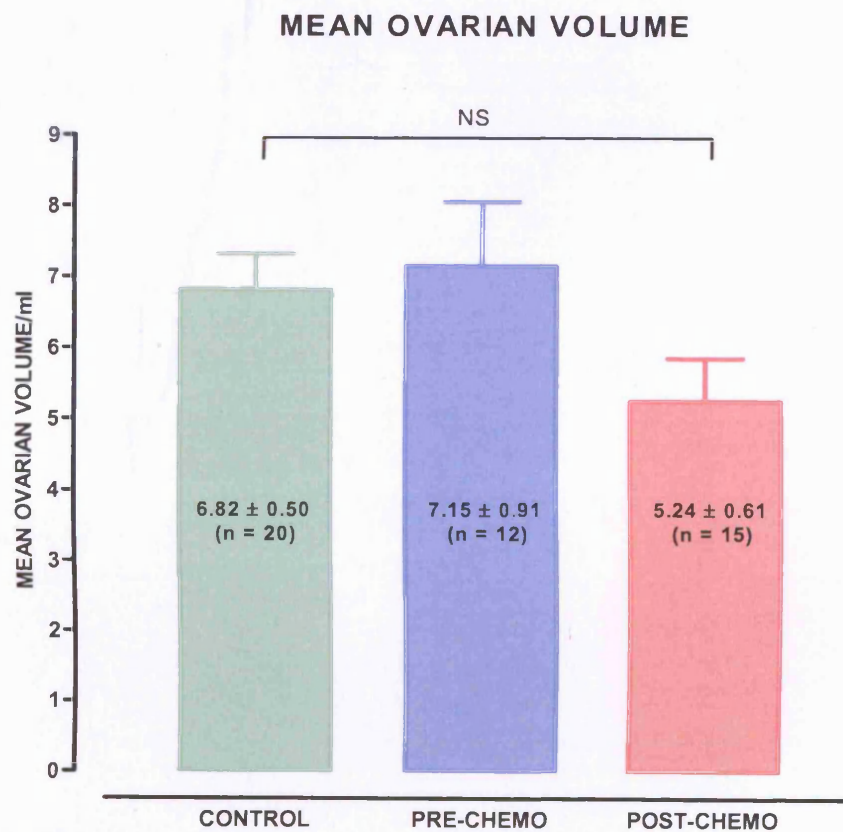


FIGURE 4.4.3.2 MOV between patients and the controls.

NS = no significant difference.

4.4.3.3 ANTRAL FOLLICLE COUNT

Analysis of variance (ANOVA) showed highly significant differences in the mean total antral follicle count (TAFC) among the three groups ($p < 0.0001$). While no significant difference was detected between the controls and the pre-chemotherapy group (95% CI -4.129 to 4.691; $p > 0.05$), there were significant differences between the controls and the post-chemotherapy group (95% CI 5.336 to 13.16; $p < 0.001$), as well as between the pre-and post-chemotherapy groups (95% CI 4.559 to 13.38; $p < 0.001$). A similar picture was seen with the mean antral follicle count (MAFC) among the three groups ($p < 0.0001$). No significant difference was detected between the controls and the pre-chemotherapy group (95% CI -2.134 to 2.414; $p > 0.05$). Significant differences were detected however between the controls and the post-chemotherapy group (95% CI 2.082 to 6.118; $p < 0.001$) and between the pre-and post-chemotherapy groups (95% CI 1.686 to 6.234; $p < 0.001$).

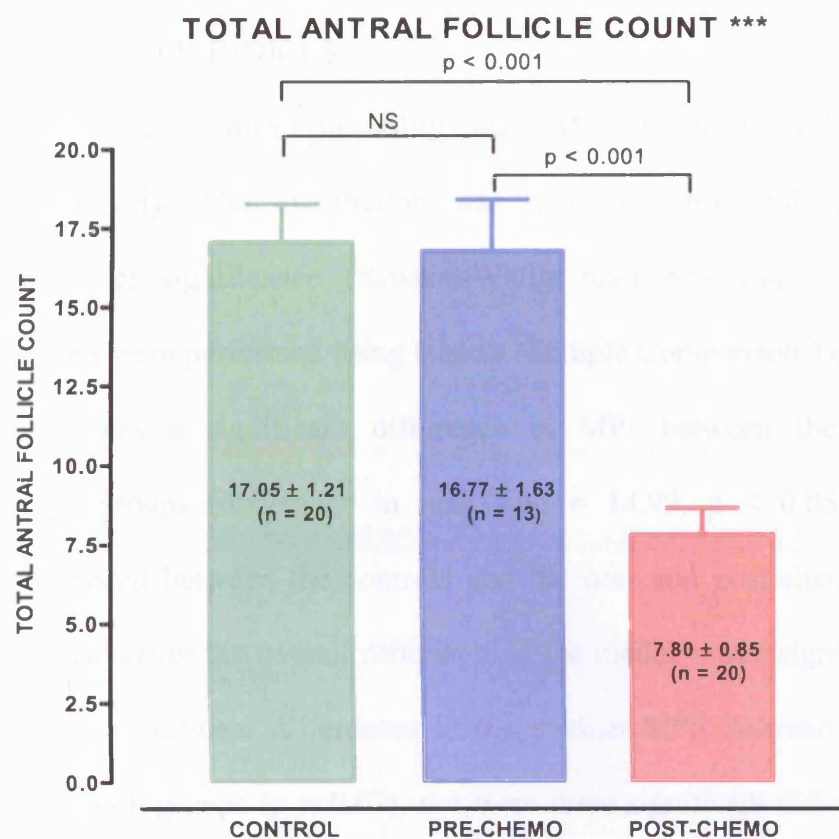


FIGURE 4.4.3.3 TAFC in all three groups.

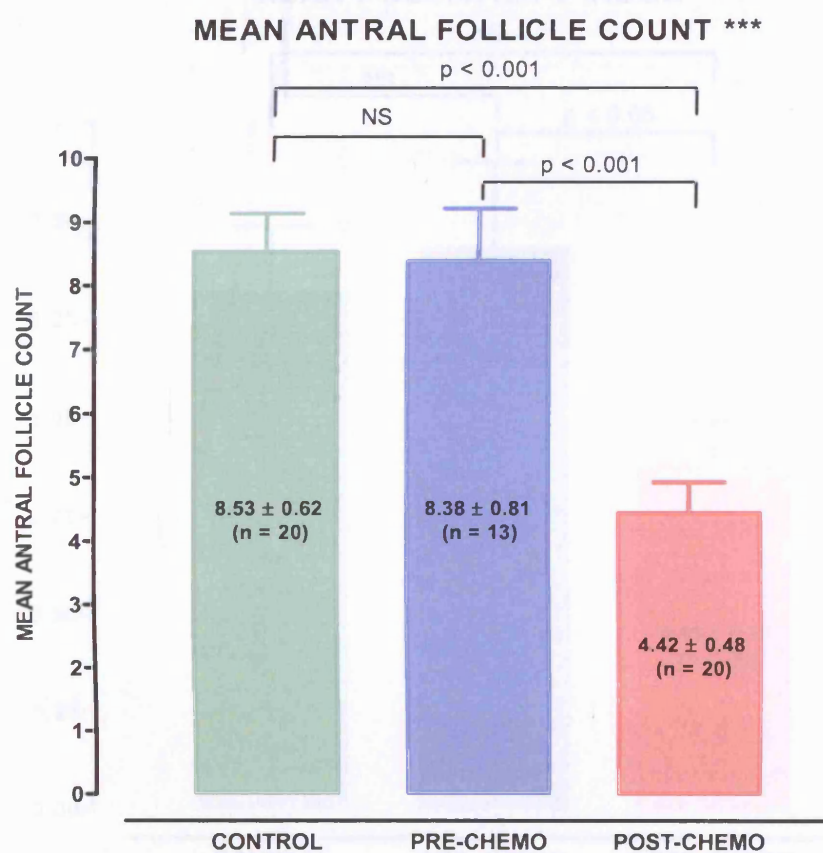


FIGURE 4.4.3.4 MAFC in all three groups.

*** p < 0.001

4.4.3.4 DOPPLER INDICES

These were assessed as mean pulsatility index (MPI) and mean peak systolic velocity (MPS) respectively. The distribution was non-parametric and as such gaussian approximation of significance (Kruskal-Wallis test) was employed. Comparisons between groups were performed using Dunn's Multiple Comparison Test.

There was a significant difference in MPI between the pre- and post-chemotherapy groups (difference in rank sum = 14.99, $p < 0.05$). No significant differences existed between the controls and the pre- and post-chemotherapy groups respectively. However the overall difference in the medians was significant ($p = 0.02$). There were no significant differences in the median MPS between the controls and patients from both groups ($p = 0.02$), nor were there significant differences within the groups.

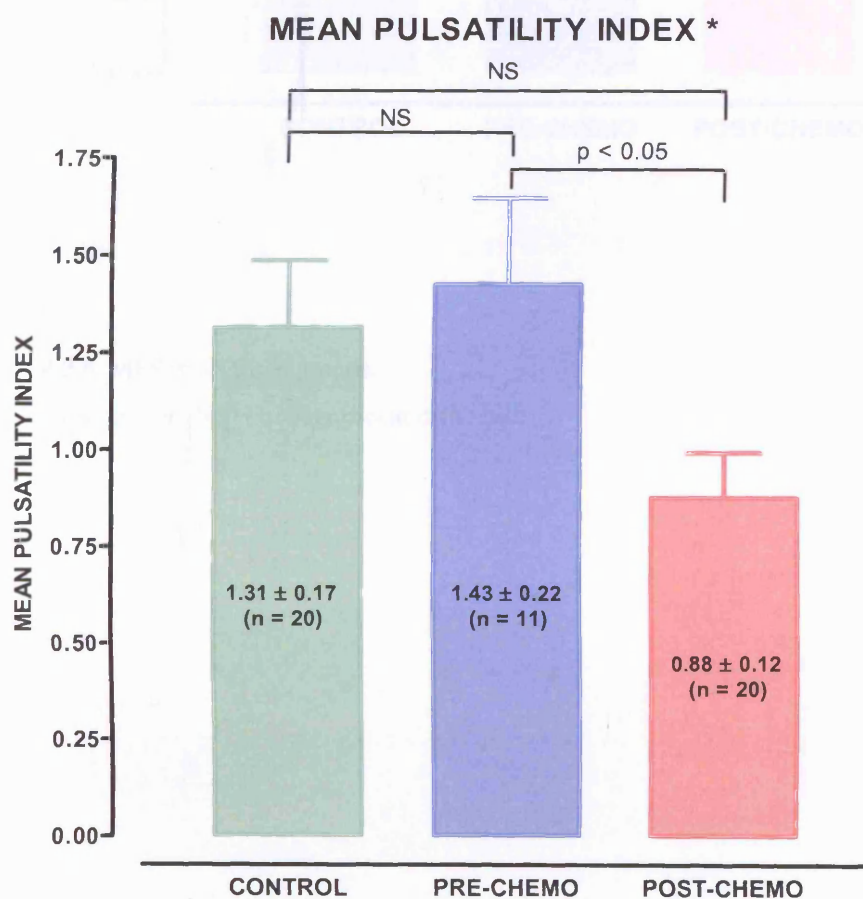


FIGURE 4.4.3.5 MPI in all three groups.

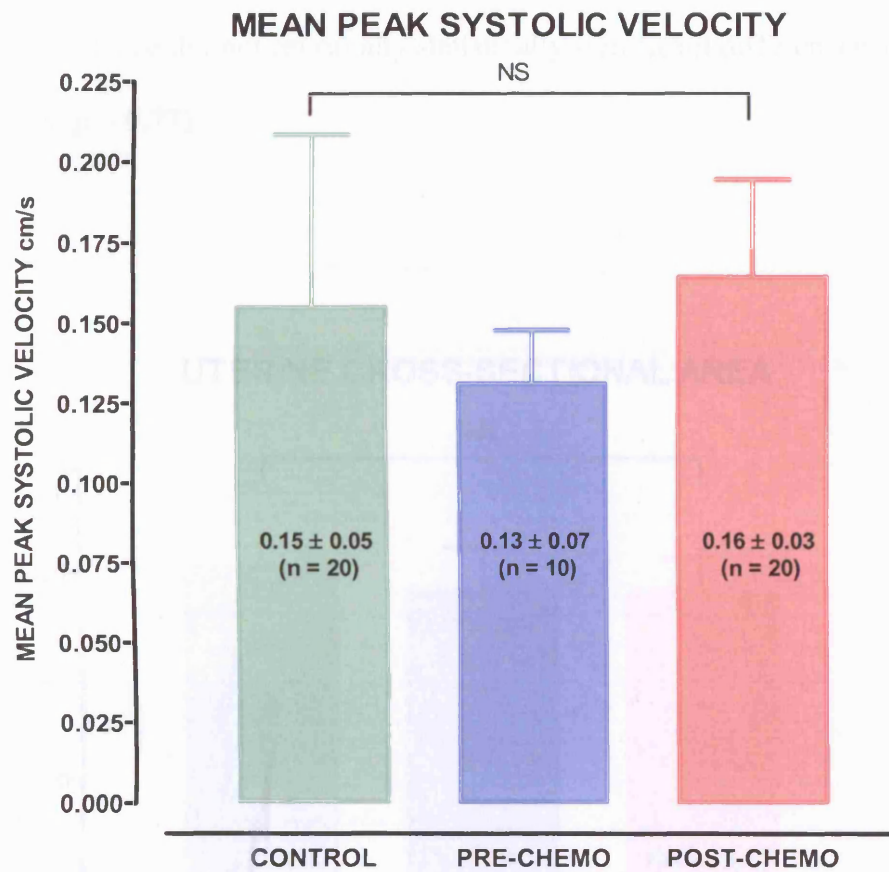


FIGURE 4.4.3.6 MPS in all three groups.

Gaussian approximation. NS = no significant difference.

4.4.3.5 UTERINE DIMENSIONS

UTERINE CROSS-SECTIONAL AREA:

Analysis of variance did not reveal any statistically significant differences among the three groups ($p = 0.77$).

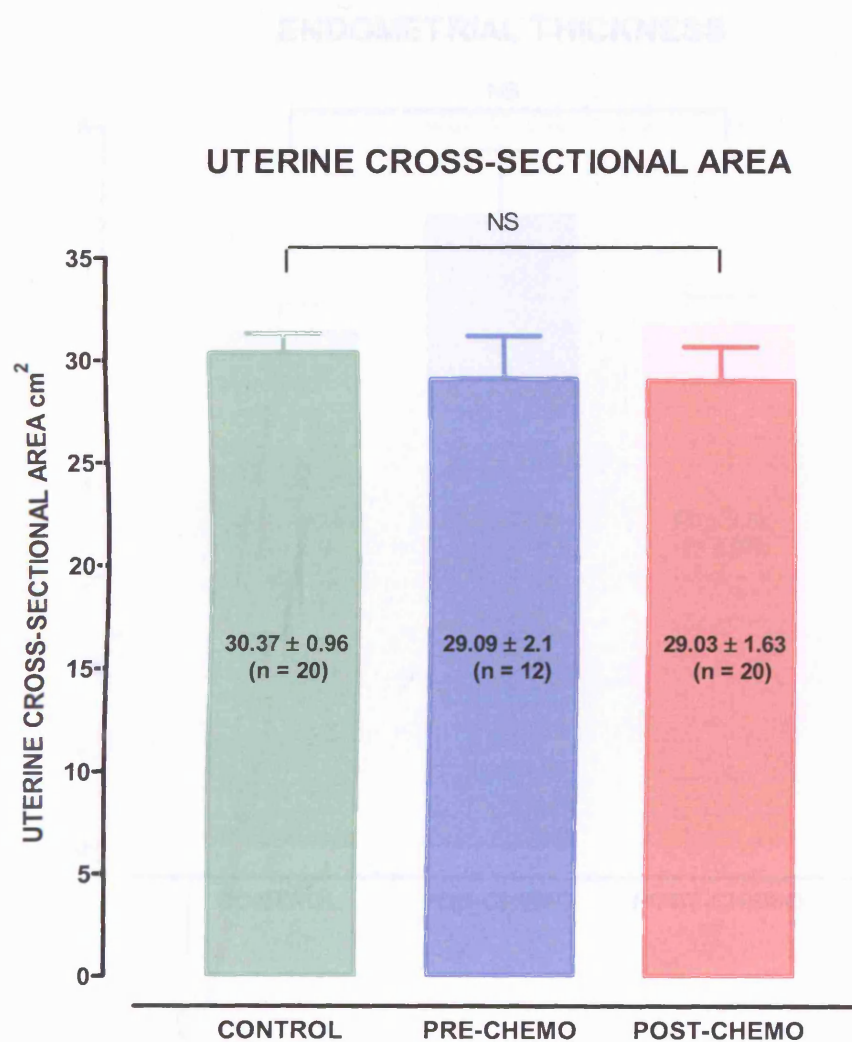


FIGURE 4.4.3.7 Uterine CSA in all three groups.

NS = no significant difference.

ENDOMETRIAL THICKNESS: DATA

Analysis of variance did not reveal any statistically significant differences among the three groups ($p = 0.09$).

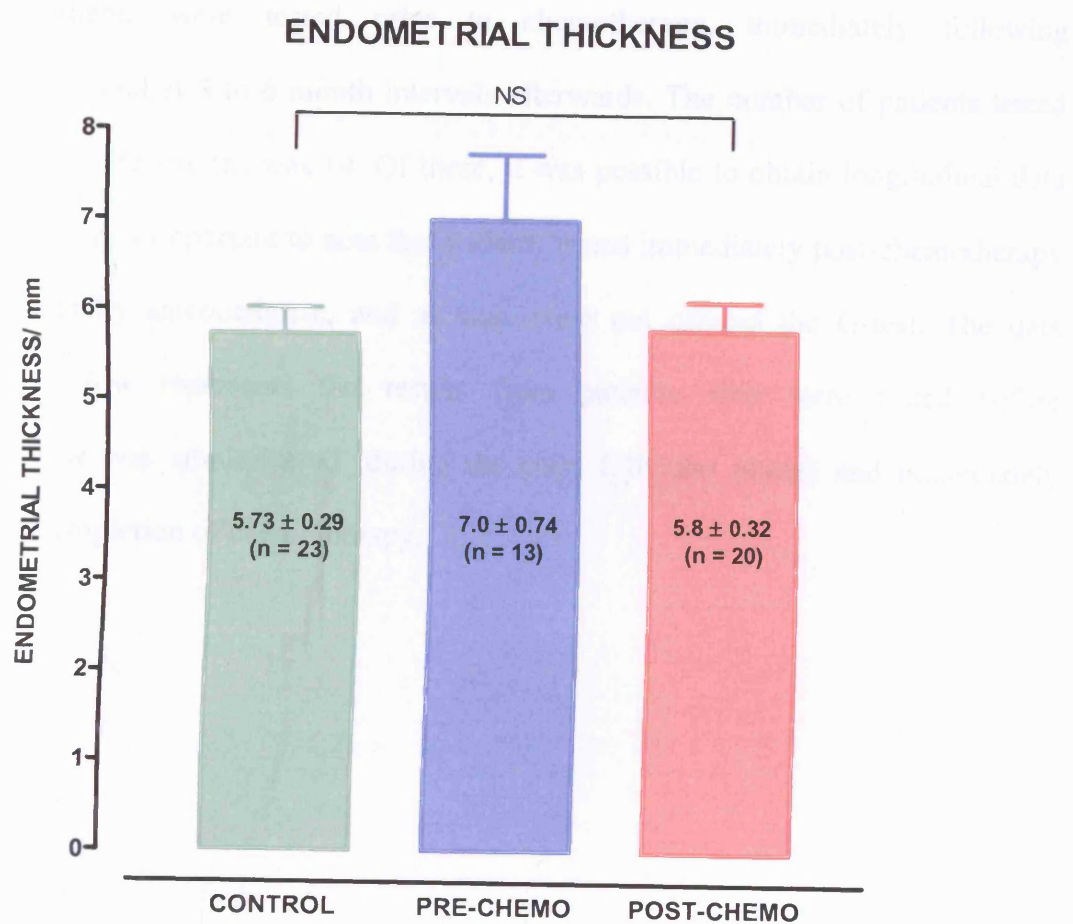


FIGURE 4.4.3.8 Uterine ET in all three groups.

NS = no significant difference.

4.5 PROSPECTIVE DATA (1)

4.5.1 INTRODUCTION

The longitudinal data presented was limited due to an unexpected freezer shutdown over the Christmas period. However, some useful information was still obtainable. In this dataset, patients were tested prior to chemotherapy, immediately following chemotherapy and at 3 to 6 month intervals afterwards. The number of patients tested prior to chemotherapy (n) was 14. Of these, it was possible to obtain longitudinal data on 8 patients. It is important to note that patients tested immediately post-chemotherapy were universally amenorrhoeic, and as such were not offered the G-test. The data displayed below represents the results from patients who were tested before chemotherapy was administered (during the early follicular phase) and immediately following completion of chemotherapy.

PT.	AGE/ YEARS	PARITY	CYCLE/ days	BMI (kg/m ²)	HISTOLOGICAL DIAGNOSIS	CHEMOTHERAPY REGIME	CUMULATIVE DOSE
1	39	0+0	5/32	25	23 mm G3 ductal ca; ER/PR moderately pos, HER2 3+ 0/7 nodes pos	FEC X 6	F = 5760mg E = 576mg C = 5760mg
2	35	0+0	7/30	23	50mm G2/3 ductal ca; ER weakly pos, 2/18 nodes pos	FEC X 6	F = 6600mg E = 660mg C = 6600mg
3	32	0+0	4/26	24	18mm G3 ductal ca, weakly ER pos 1/14 nodes pos	EC X 4 Paclitaxel X 4 Gemcitabine X 4	E = 688mg C = 4400mg P = 1392mg G = 19152mg
4	35	0+0	5/30	30	25 mm G2 ductal ca; ER/PR neg; her2 3+; 0/8 nodes pos	FEC X 6	F = 6480mg E = 648mg C = 6480mg
5	33	0+0	5/30	21	G3 ductal ca, ER/PR neg, HER2 1+	FEC X 6	F = 5810mg E = 596mg C = 5810mg
6	41	1+1	5/28	26	42mm high grade DCIS with 2 invasive foci G2; ER/PR neg; 2/12 nodes pos	ECx4 paclitaxelx4	E = 702mg C = 4700mg P = 1368mg herceptin
7	31	0+1	3/34	20	G3 Invasive ductal ca; ER/PR neg; HER2 3+	ECx4 paclitaxelx4	E = 564mg C = 3740mg P = 1074mg herceptin
8	38	0+0	6/28	25	Grade 2 ductal carcinoma, ER+ve, HER2-ve	Ex4 CMFx4	E = 8284mg C = 9920mg M = 640mg F = 9600mg

TABLE 4.5.1 CLINICAL DATA OF PATIENTS IN LONGITUDINAL GROUP

The patients in this group comprised the longitudinal arm of the study, with all patients having ovarian reserve tests performed prior to receiving chemotherapy. Cycle length is depicted first by menstruation length followed by cycle length in days. As per the study protocol, all patients in this group had ovarian reserve tests performed between cycle days 2 – 5. Relevant details of diagnosis and chemotherapy regimen (including cumulative dosages) are shown.

BMI = body mass index, G = grade, ER = oestrogen receptor status, PR = progesterone receptor status, HER2 = HER2 receptor status, Pos = positive, neg = negative, E = epirubicin, C = cyclophosphamide, M = methotrexate, F = fluorouracil, P = paclitaxel, G = gemcitabine

4.5.2 BIOCHEMICAL RESULTS

4.5.2.1 BASAL FSH

Basal FSH levels were significantly different between groups (number of pairs = 8). The mean of the differences was = - 38.85 (95% CI -63.62 to -14.08), with an overall $p = 0.008$.

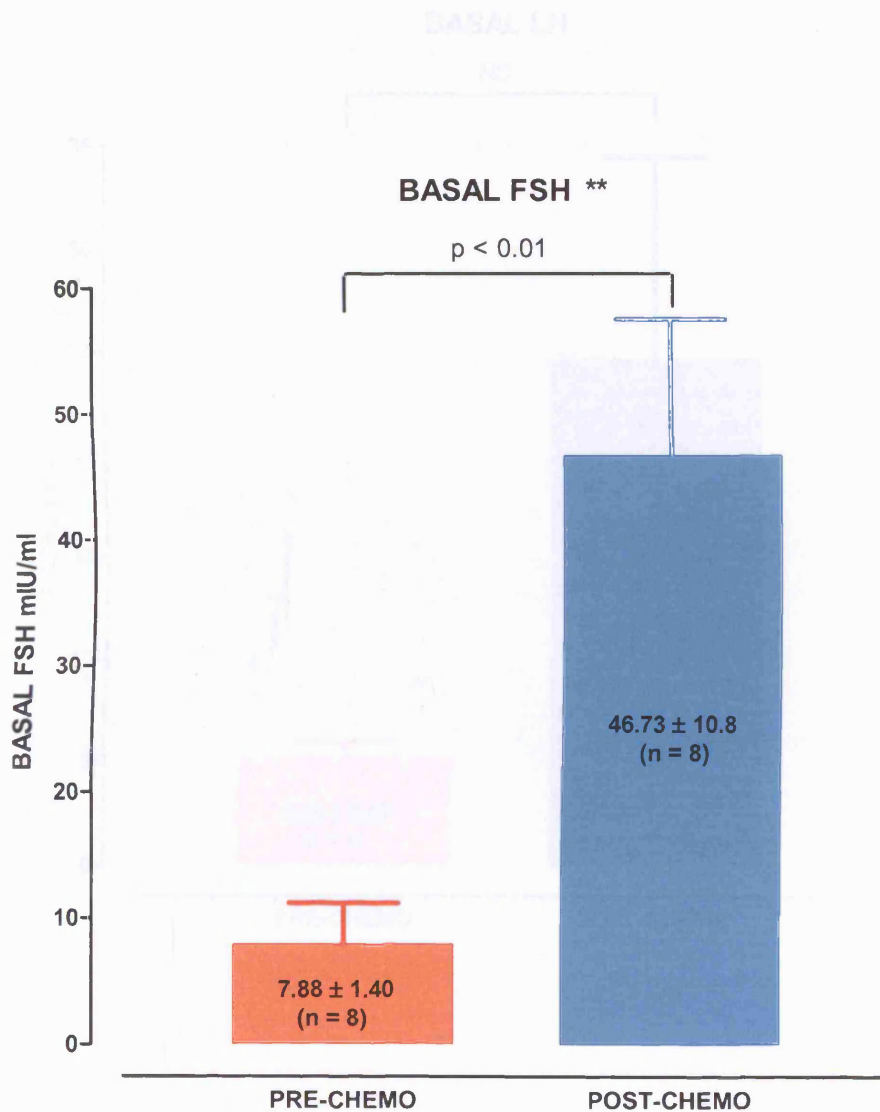


FIGURE 4.5.2.1 Basal FSH levels pre vs. post chemotherapy in 8 patients.

** $p < 0.01$

4.5.2.2 BASAL LH

Basal LH levels were not significantly different between groups (number of pairs = 6).

The mean of the differences was = -19.44 (95% CI -46.41 to 7.526), with an overall p = 0.12.

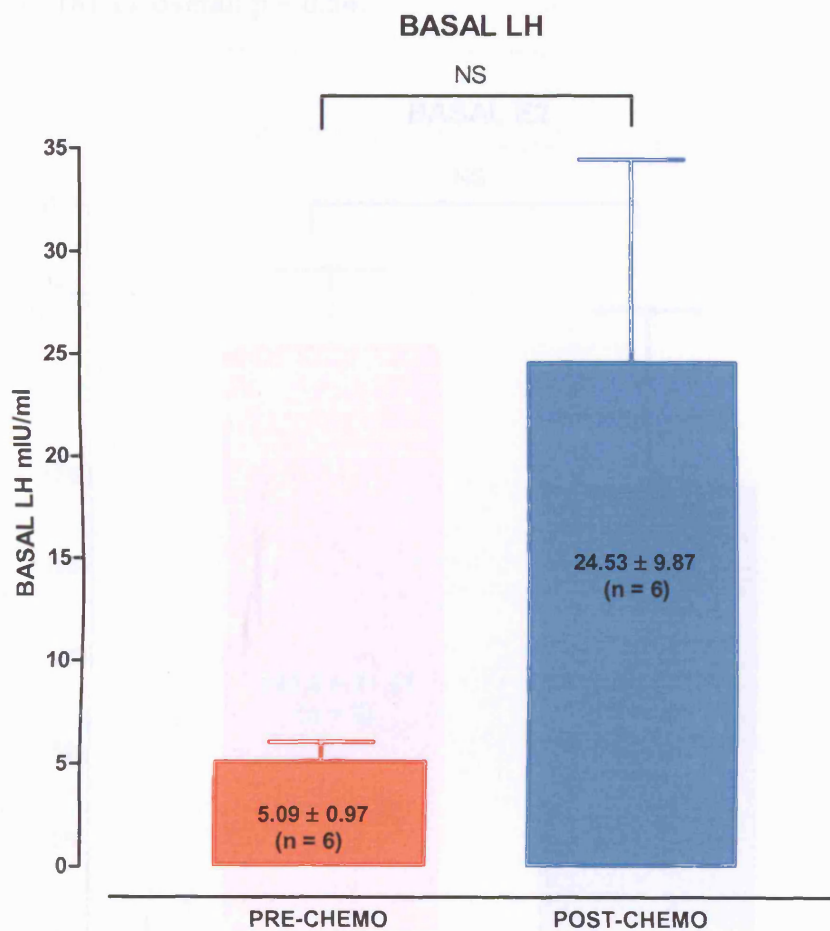


FIGURE 4.5.2.2 Basal LH levels pre vs. post chemotherapy in 6 patients.

NS = no significant difference.

4.5.2.3 BASAL E2

Although the mean basal oestradiol levels were lower in patients following chemotherapy, the difference between pre- and post-chemotherapy levels was not statistically significant. The mean of differences in the 8 pairs analyzed was 38.74 (95% CI -103.6 to 181.1), overall $p = 0.54$.

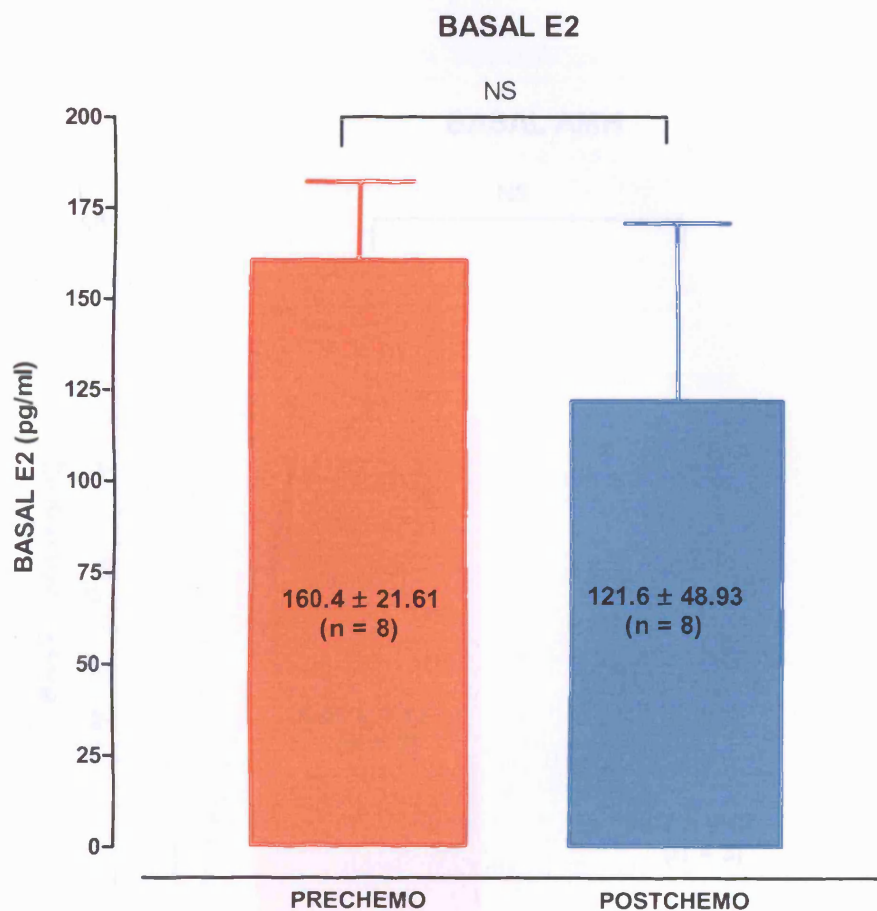


FIGURE 4.5.2.3 Basal oestradiol levels in 8 patients pre and post-chemotherapy.

NS = no significant difference.

4.5.2.4 BASAL AMH

For reasons described previously, there were a limited number of samples available for analysis in this group (number of pairs = 3). As such, the distribution was assumed to be non-gaussian and the Wilcoxon signed rank test used for analysis. There was a large difference in bAMH levels pre vs. post chemotherapy, but this was not statistically significant.

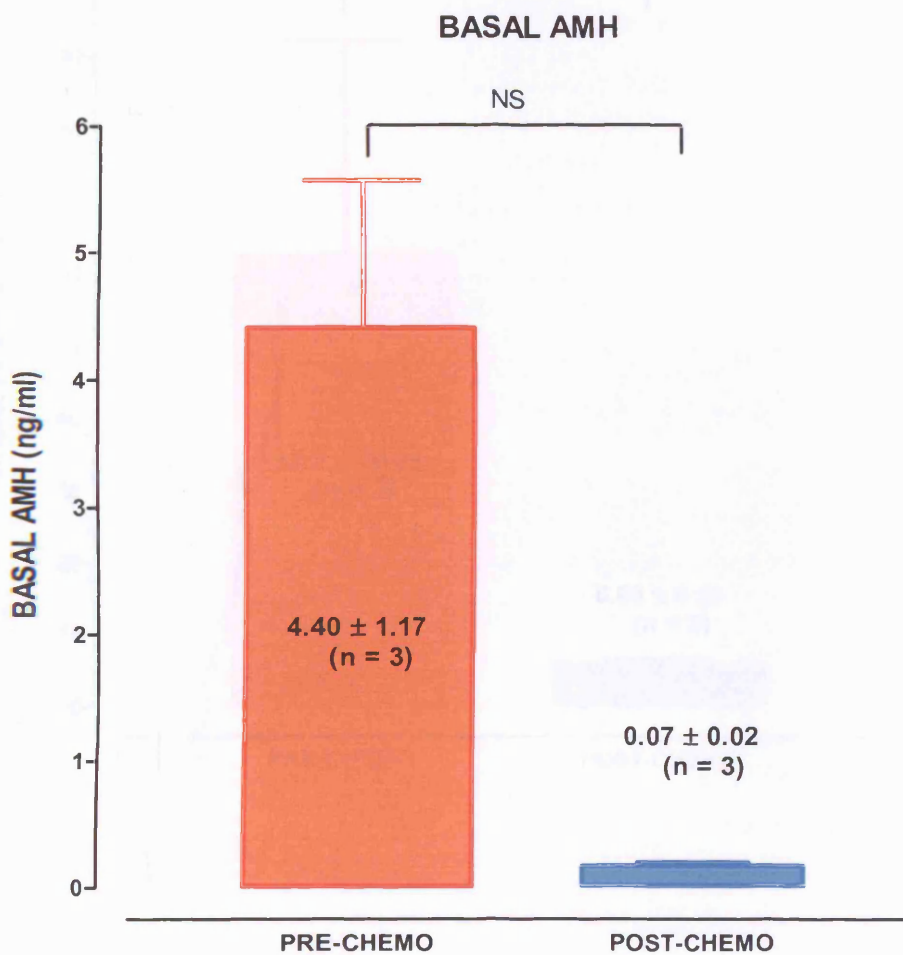


FIGURE 4.5.2.4 Basal AMH levels in 3 patients pre and post-chemotherapy.

NS = no statistically significant difference.

4.5.2.5 BASAL INHIBIN B

As was the case with AMH, a statistically significant difference between pre- and post-chemotherapy levels of inhibin B could not be determined due to the small number of samples available (number of pairs = 3).

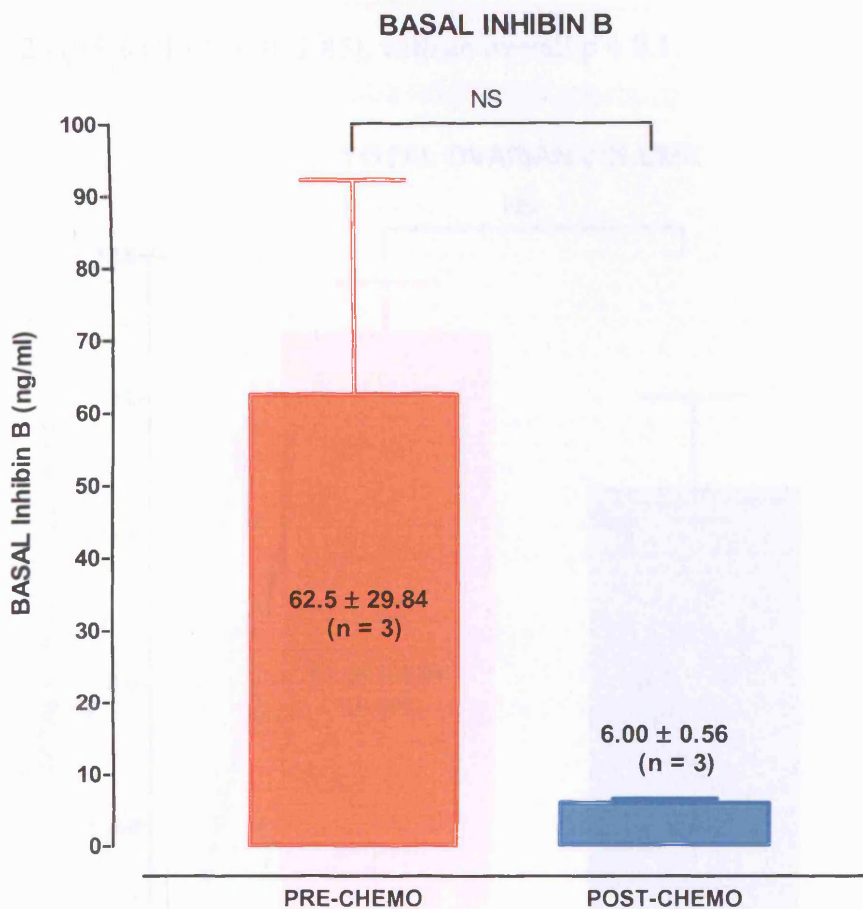


FIGURE 4.5.2.5 Basal Inhibin B levels in 3 patients pre and post-chemotherapy.

NS = no statistically significant difference.

4.5.3 BIOPHYSICAL PARAMETERS

4.5.3.1 OVARIAN VOLUME

Total (TOV) and mean (MOV) ovarian volumes were not significantly different between groups (number of pairs = 7). For TOV the mean of the differences was 2.71 (95% CI -0.67 to 6.1), with an overall $p = 0.1$. For MOV, the mean of the differences was = 1.25 (95% CI -0.34 to 2.85), with an overall $p = 0.1$.

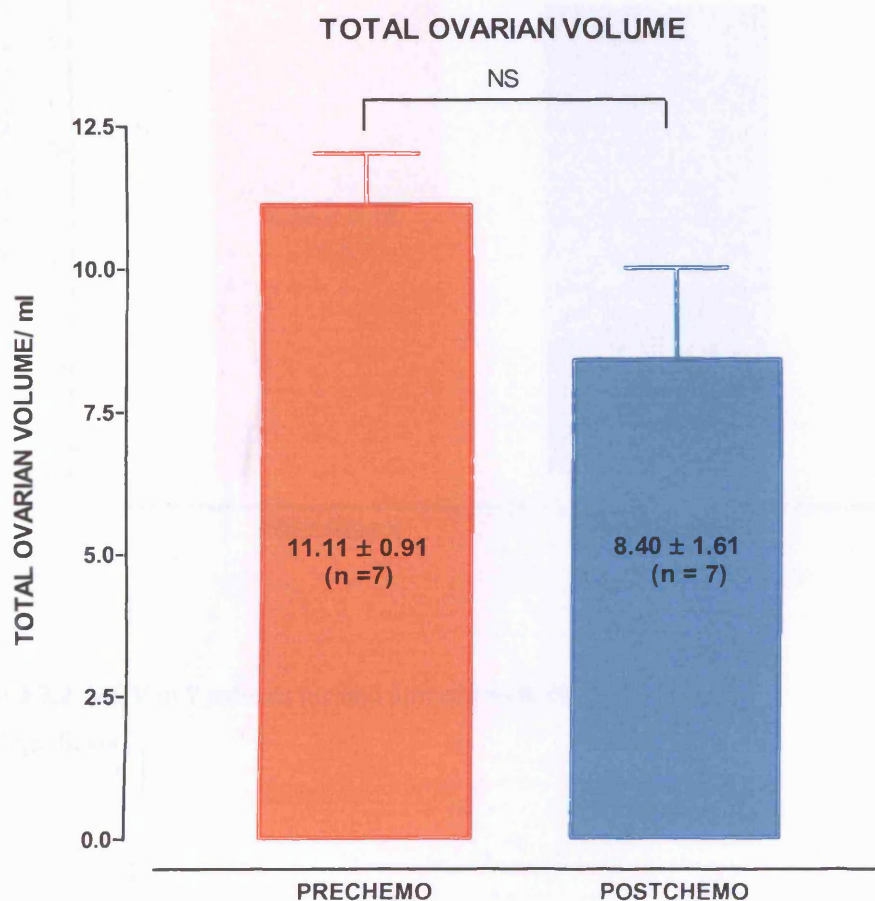


FIGURE 4.5.3.1 TOV in 7 patients pre and post-chemotherapy.

NS = not significant

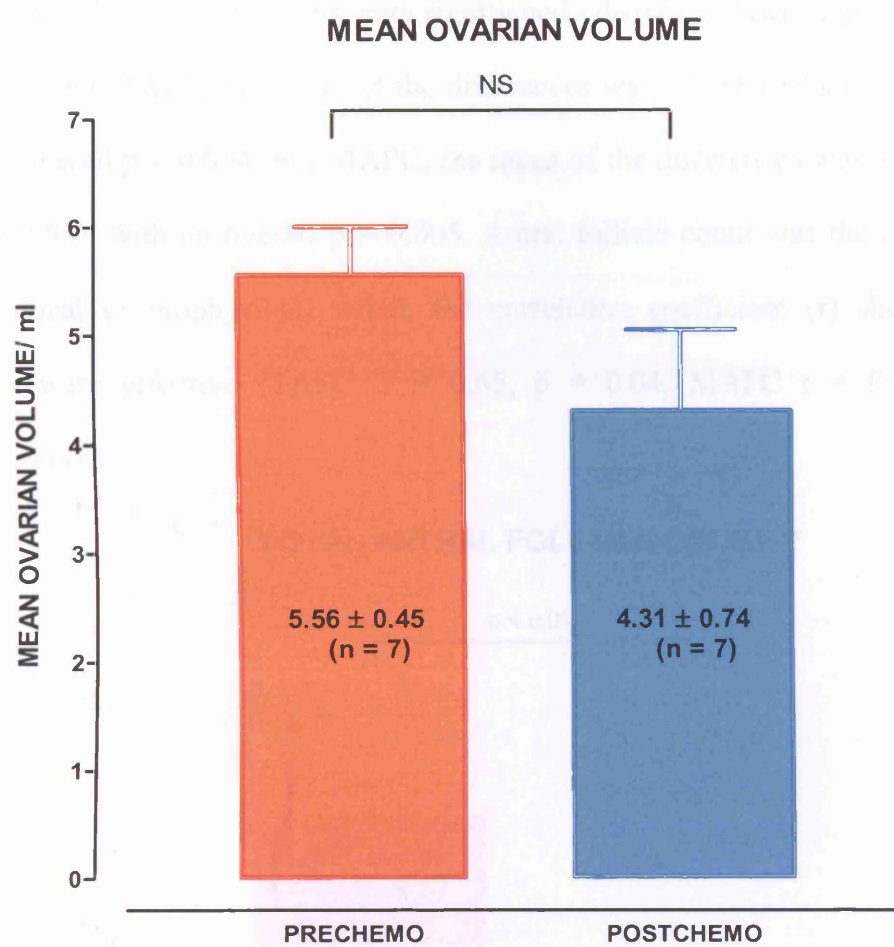


FIGURE 4.5.3.2 MOV in 7 patients pre and post-chemotherapy.

NS = not significant.

4.5.3.2 ANTRAL FOLLICLE COUNT

Both total and mean AFC levels were significantly different between groups (number of pairs = 8). For TAFC, the mean of the differences was = 9.88 (95% CI 4.47 to 15.28), with an overall $p = 0.004$. For MAFC, the mean of the differences was = 4.81 (95% CI 2.04 to 7.59), with an overall $p = 0.005$. Antral follicle count was the only parameter (biochemical or biophysical) where the correlation coefficient (r) showed that the pairings were effective (TAFC: $r = 0.65$, $p = 0.04$, MAFC $r = 0.65$, $p = 0.04$ respectively).

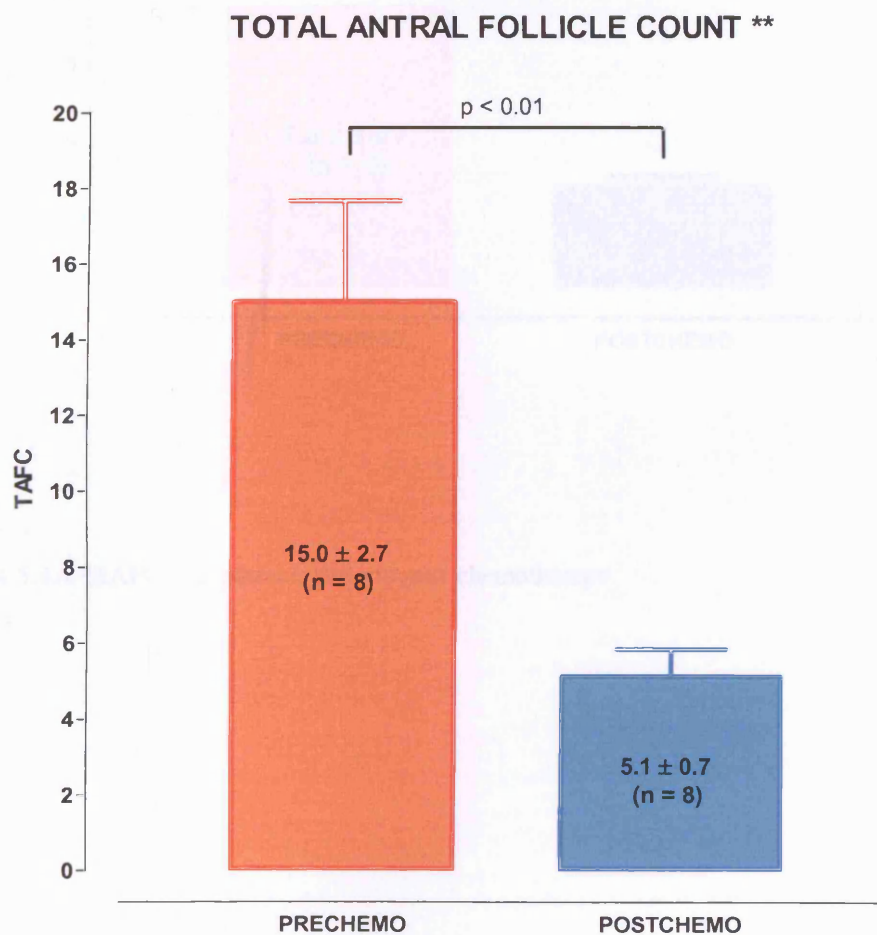


FIGURE 4.5.3.3 TAFC in 8 patients' pre and post-chemotherapy.

** $p < 0.01$.

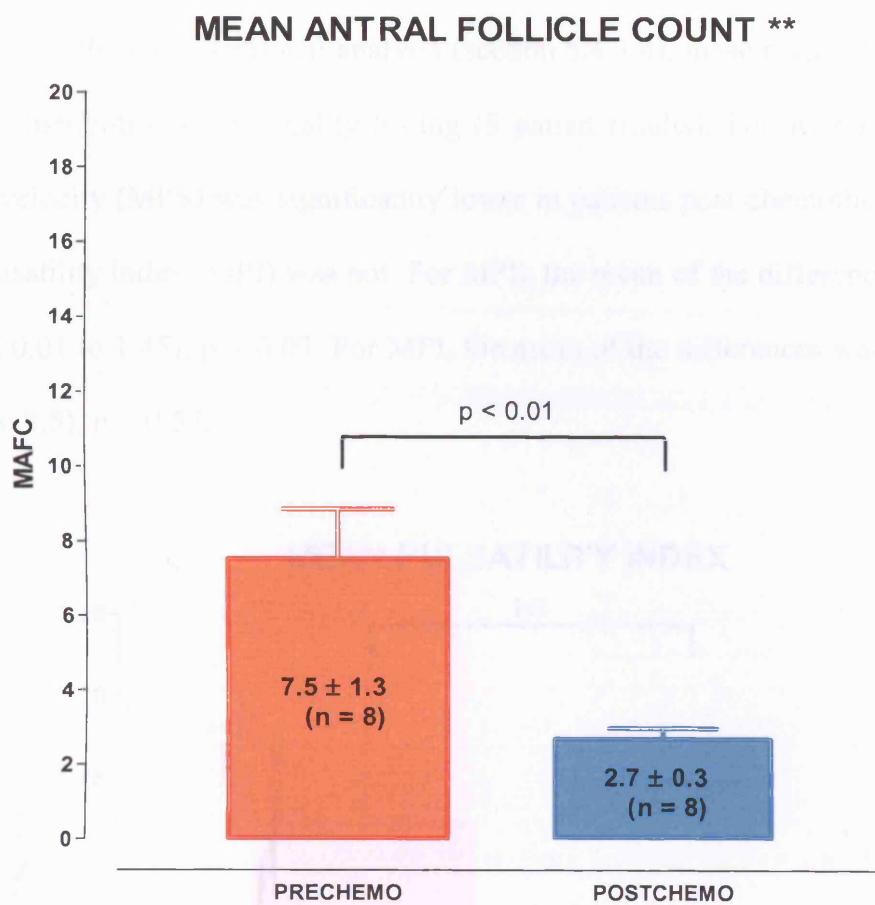


FIGURE 4.5.3.4 MAFC in 8 patients pre and post-chemotherapy.

** p < 0.01

4.5.3.3 DOPPLER INDICES

In contrast to the cross-sectional analysis (section 5.4.3.4), these parameters followed a gaussian distribution on normality testing (5 paired results). Furthermore, mean peak systolic velocity (MPS) was significantly lower in patients post-chemotherapy, whereas mean pulsatility index (MPI) was not. For MPS, the mean of the differences was = 0.08 (95% CI 0.01 to 1.45), $p = 0.03$. For MPI, the mean of the differences was = 0.45 (95% CI -1.6 to 2.5), $p = 0.57$.

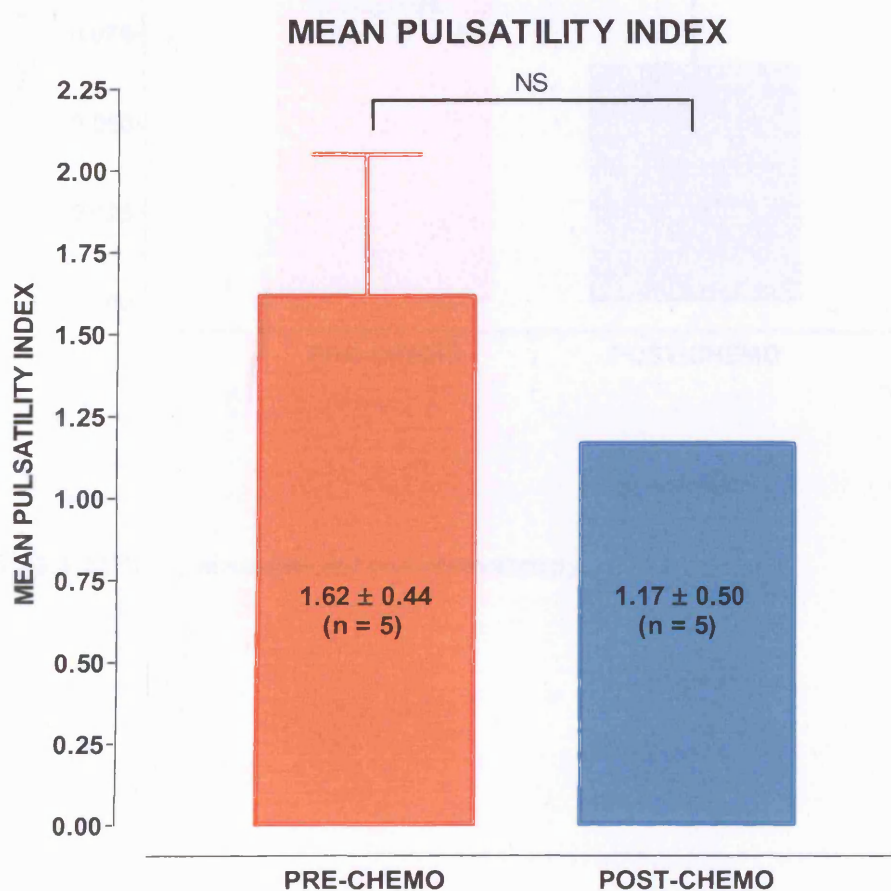


FIGURE 4.5.3.5 MPI in patients pre- and post-chemotherapy.

NS = not significant.

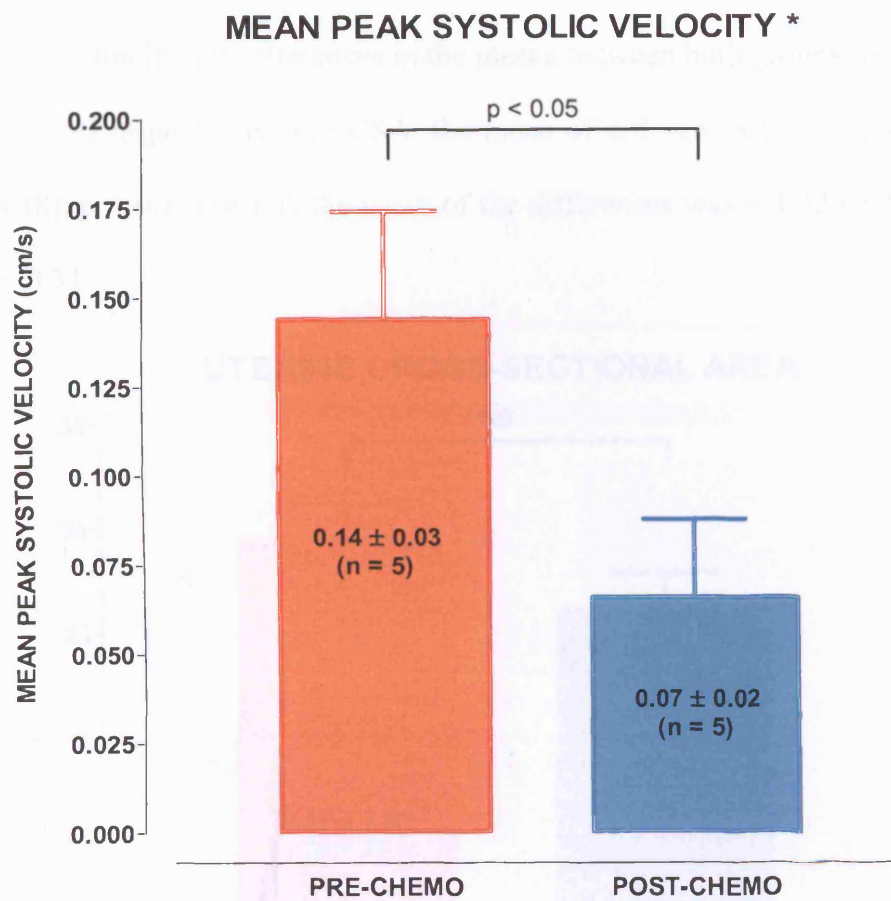


FIGURE 4.5.3.6 MPS in patients pre- and post-chemotherapy.

* $p < 0.05$

4.5.3.4 UTERINE DIMENSIONS

There were no significant differences in the means between both groups for CSA (n = 7) and ET (n = 8) respectively. For CSA, the mean of differences (m) = 3.41 (95% CI - 1.65 to 8.48), p = 0.2. For ET, the mean of the differences was = 1.32 (95%CI -1.60 to 4.25), p = 0.31.

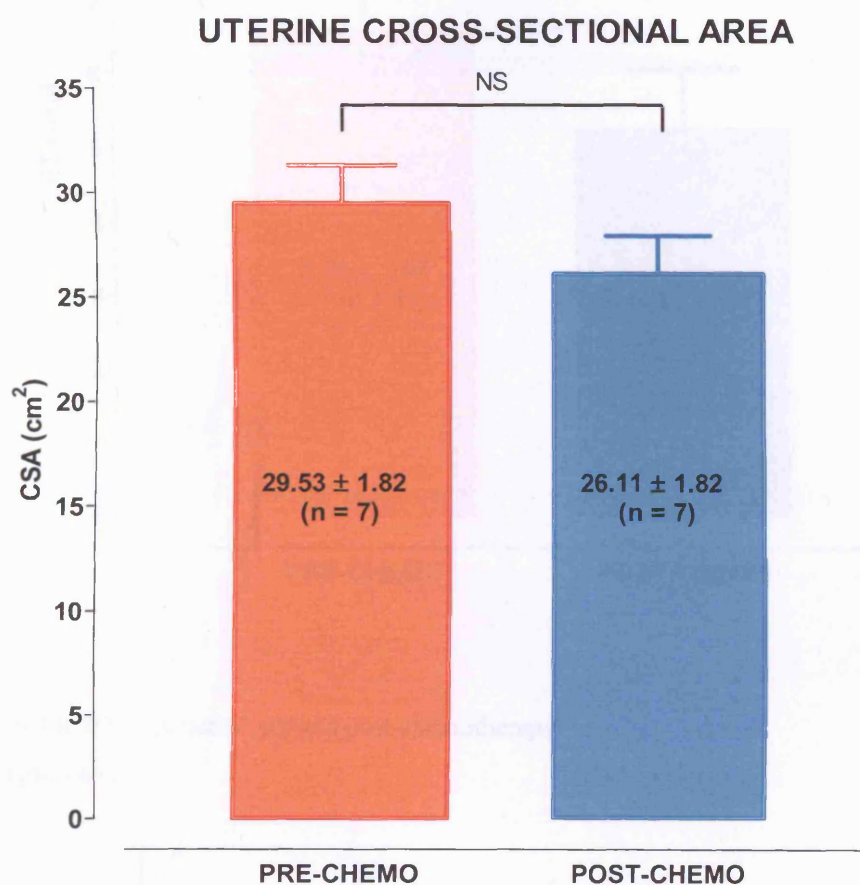


FIGURE 4.5.3.7 CSA in patients pre and post-chemotherapy.

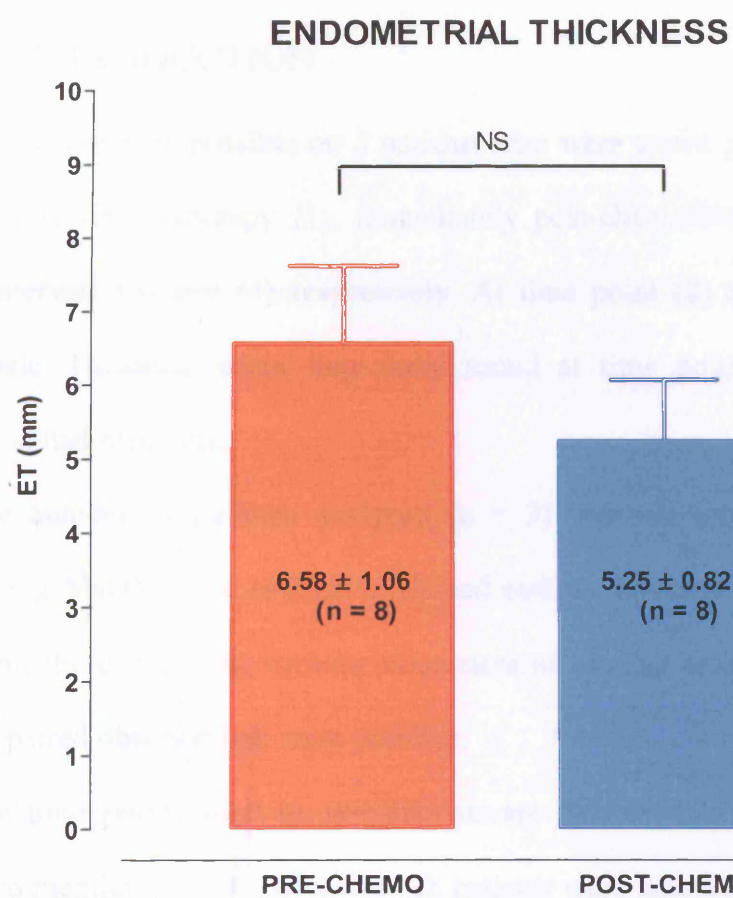


FIGURE 4.5.3.8 ET in patients' pre and post-chemotherapy.

NS = not significant.

4.6 PROSPECTIVE DATA (2)

4.6.1 INTRODUCTION

A limited analysis was possible on 3 patients who were tested prospectively at least 3 times: prior to chemotherapy (1), immediately post-chemotherapy (2), and at three monthly intervals (3) and (4) respectively. At time point (2) all three patients were amenorrhoeic. However when they were tested at time points (3) or (4), regular menstruation had resumed.

The number of patients analysed (n = 3) was too small to allow statistical analysis using ANOVA (for reasons explained earlier); however it was still informative to document the changes in various parameters of ovarian reserve where a sufficient number of paired observations were possible.

The time points used to test patients are described in the table below. It is important to mention that at time-point (2), patients were amenorrhoeic, and as such the G-test was not performed. At all other time-points however, testing was performed in the early follicular phase of the menstrual cycle.

CODE	TIME-POINT DEFINITION
1	PRE-CHEMOTHERAPY
2	IMMEDIATELY POST-CHEMOTHERAPY
3	3-MONTHS POST-CHEMOTHERAPY
4	6-MONTHS POST-CHEMOTHERAPY

TABLE 4.6.1 Definition of time points used in prospective study.

4.6.2 BIOCHEMICAL PARAMETERS

4.6.2.1 BASAL FSH

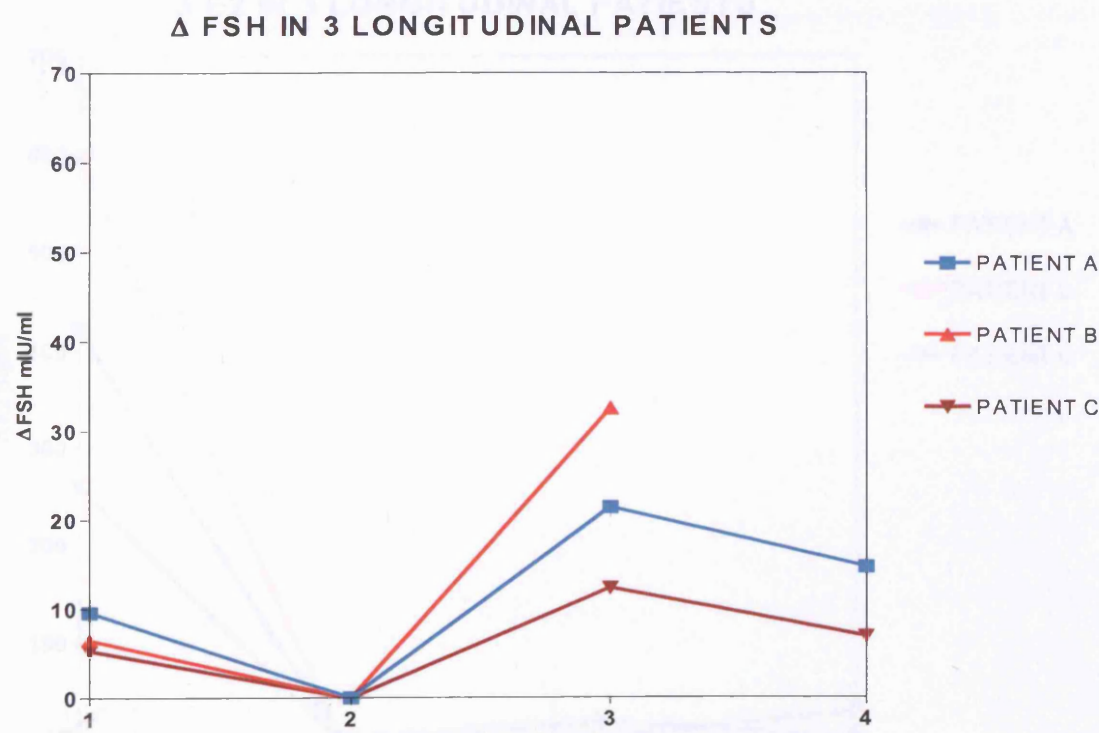
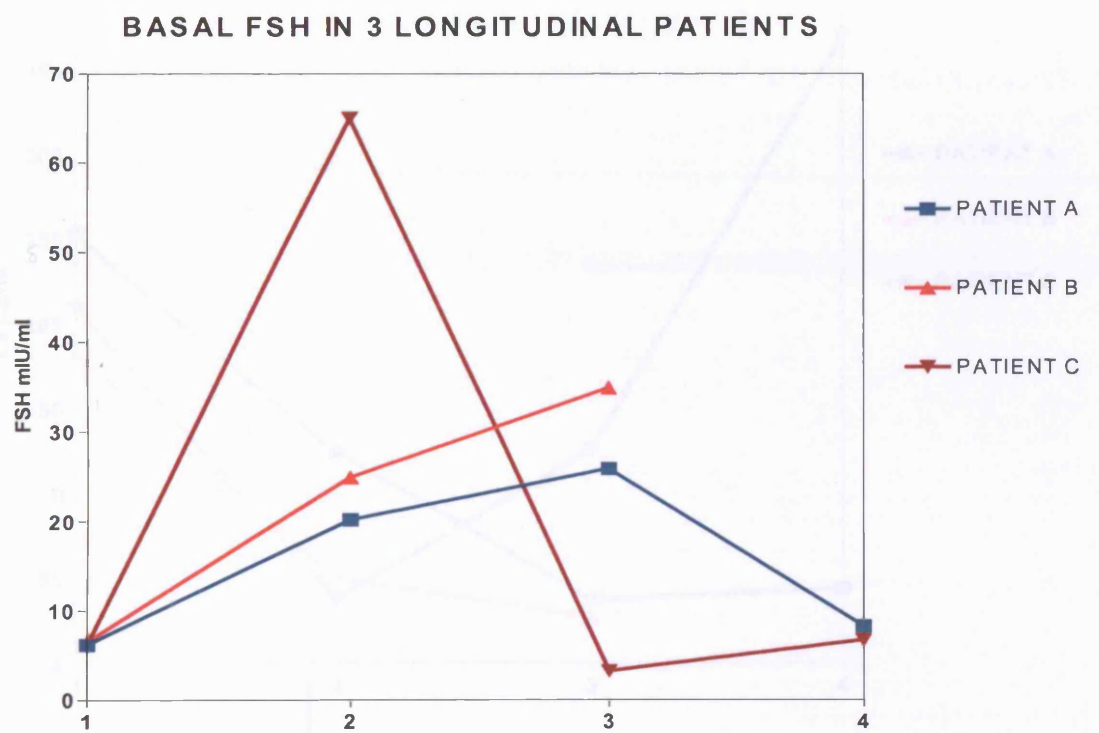


FIGURE 4.6.2.1 Basal and delta FSH in 3 patients followed prospectively. Patients were tested at 3 time points (PATIENTS B+C) and 4 time points (PATIENT A) respectively.

4.6.2.2 BASAL E2

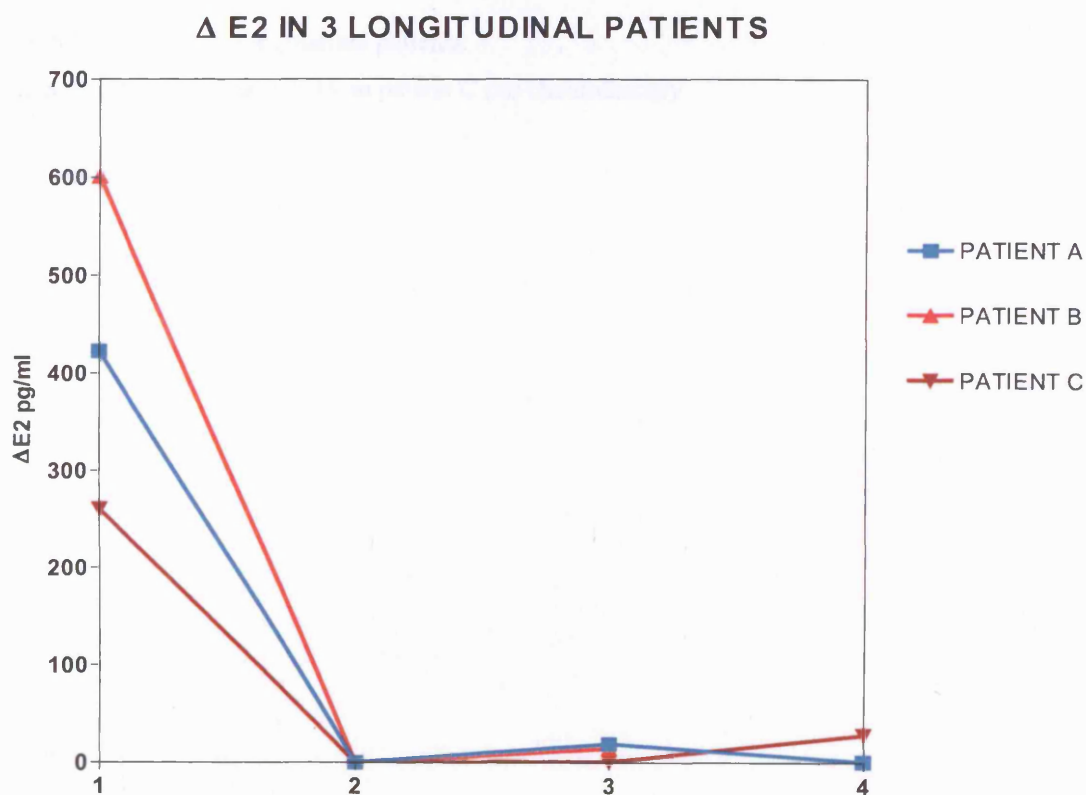
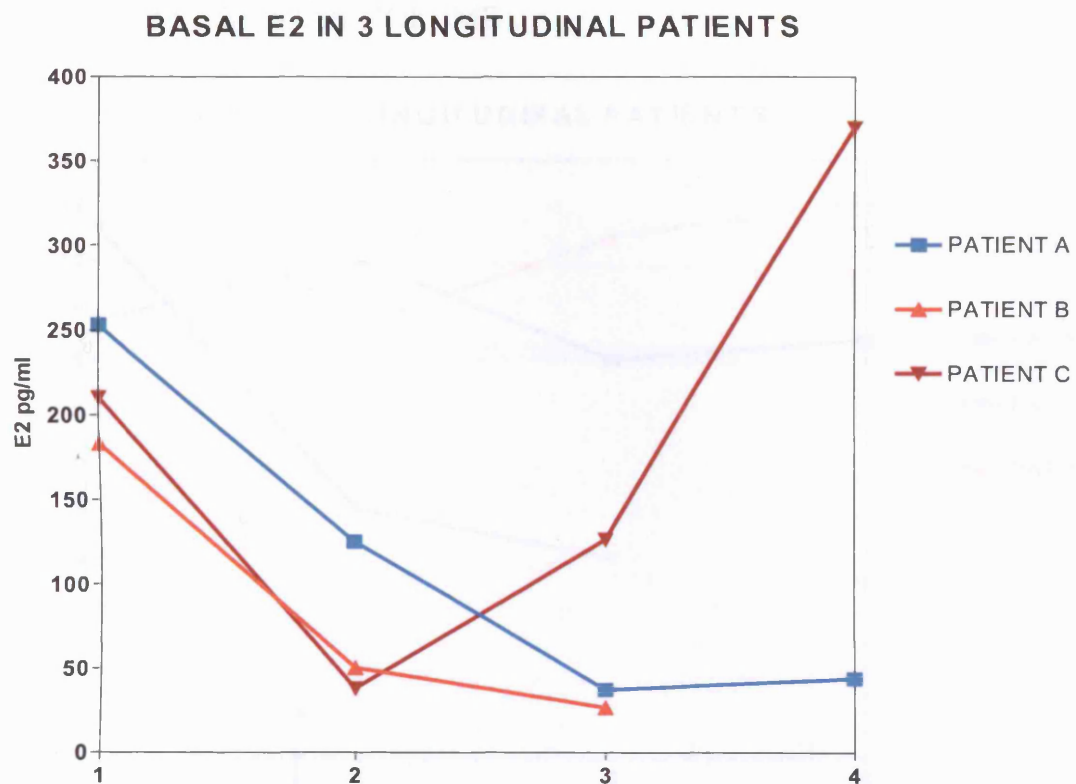


FIGURE 4.6.2.2 Oestradiol levels (basal and delta) in three patients followed prospectively.

4.6.3 BIOPHYSICAL PARAMETERS

4.6.3.1 TOTAL OVARIAN VOLUME

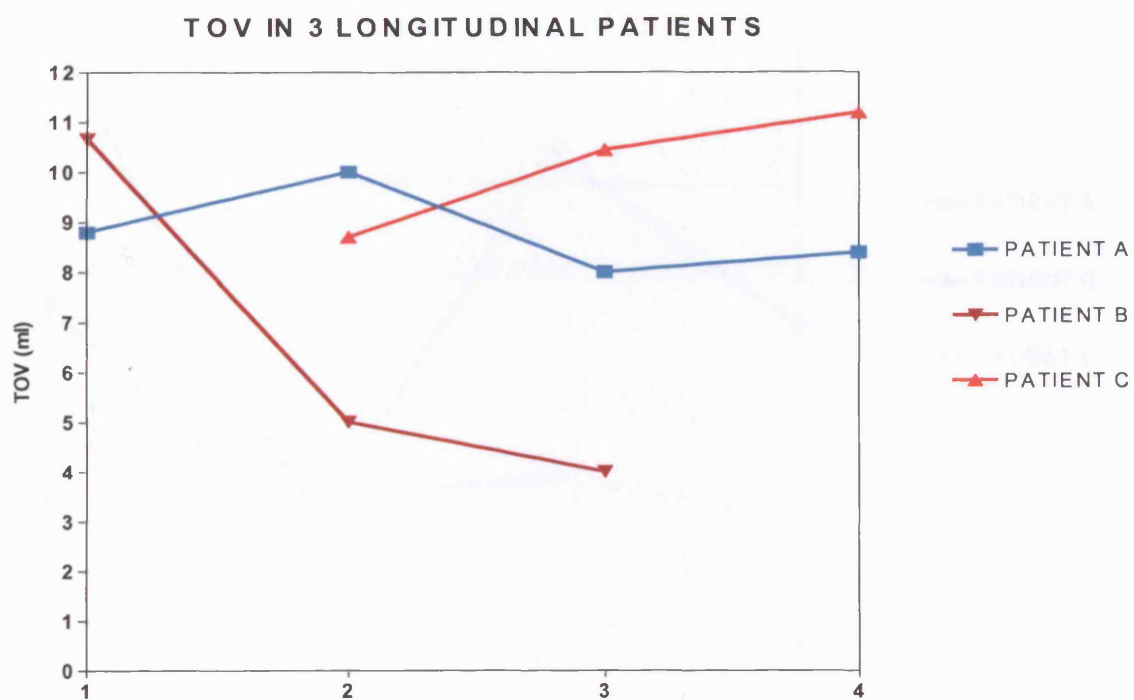


FIGURE 4.6.3.1 TOV in 3 longitudinal patients.

It was not possible to determine TOV in patient C pre-chemotherapy.

4.6.3.2 TOTAL ANTRAL FOLLICLE COUNT

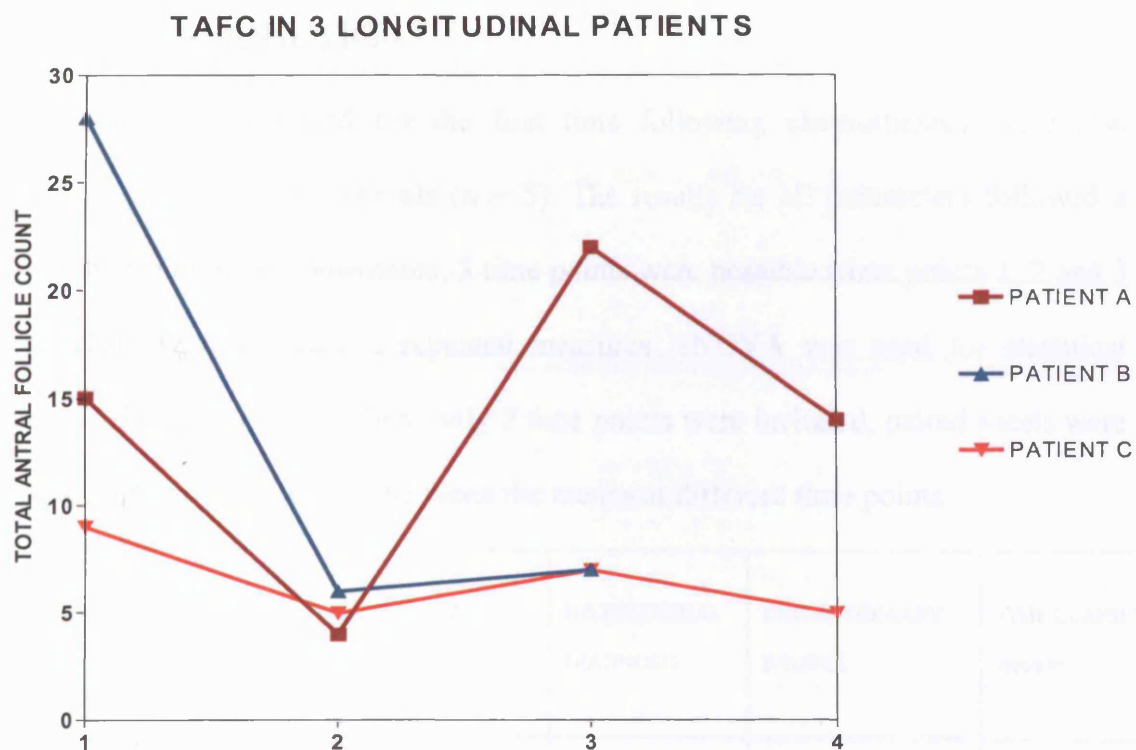


FIGURE 4.6.3.2 Total antral follicle count in the same 3 patients followed up prospectively.
The mean values give almost identical plots.

4.7 PROSPECTIVE DATA (3)

4.7.1 INTRODUCTION

Patients that were recruited for the first time following chemotherapy were also followed up at 3-monthly intervals (n = 5). The results for all parameters followed a gaussian distribution. In some cases, 3 time points were possible (time points 1, 2 and 3 respectively), in which case a repeated measures ANOVA was used for statistical analysis. In the rest of cases where only 2 time points were included, paired t-tests were used to identify any differences between the means at different time points.

AGE / years	PARITY	BMI kg/m ²	HISTOLOGICAL DIAGNOSIS	CHEMOTHERAPY REGIME	TIME ELAPSED /months
33	0+0	24	20mm G3 IDCA. ER/PR neg	E-CMF X 8	37
38	0+1	28	19mm G3 IDCA ER/PR neg	D X 4 FEC X 4	8
37	0+1	21	25mm G3 IDCA ER/PR neg	FEC X 6	58
37	1+0	24	60mm G3 IDCA ER/PR neg	D X 4 E-CMF X 6	54
36	0+0	23	85mm G3 IDCA ER/PR neg	FEC X 6 D X 2	15

TABLE 4.7.1 Details of patients recruited post-chemotherapy and tested prospectively.

G = grade IDCA = invasive ductal carcinoma, E = epirubicin, C = cyclophosphamide, M = methotrexate,

F = 5-Fluorouracil, D = docetaxel

4.7.2 BIOCHEMICAL PARAMETERS

4.7.2.1 FOLLICLE STIMULATING HORMONE

There were no significant differences between the three times tested for basal, stimulated or delta FSH as determined by Bonferroni's multiple comparison tests.

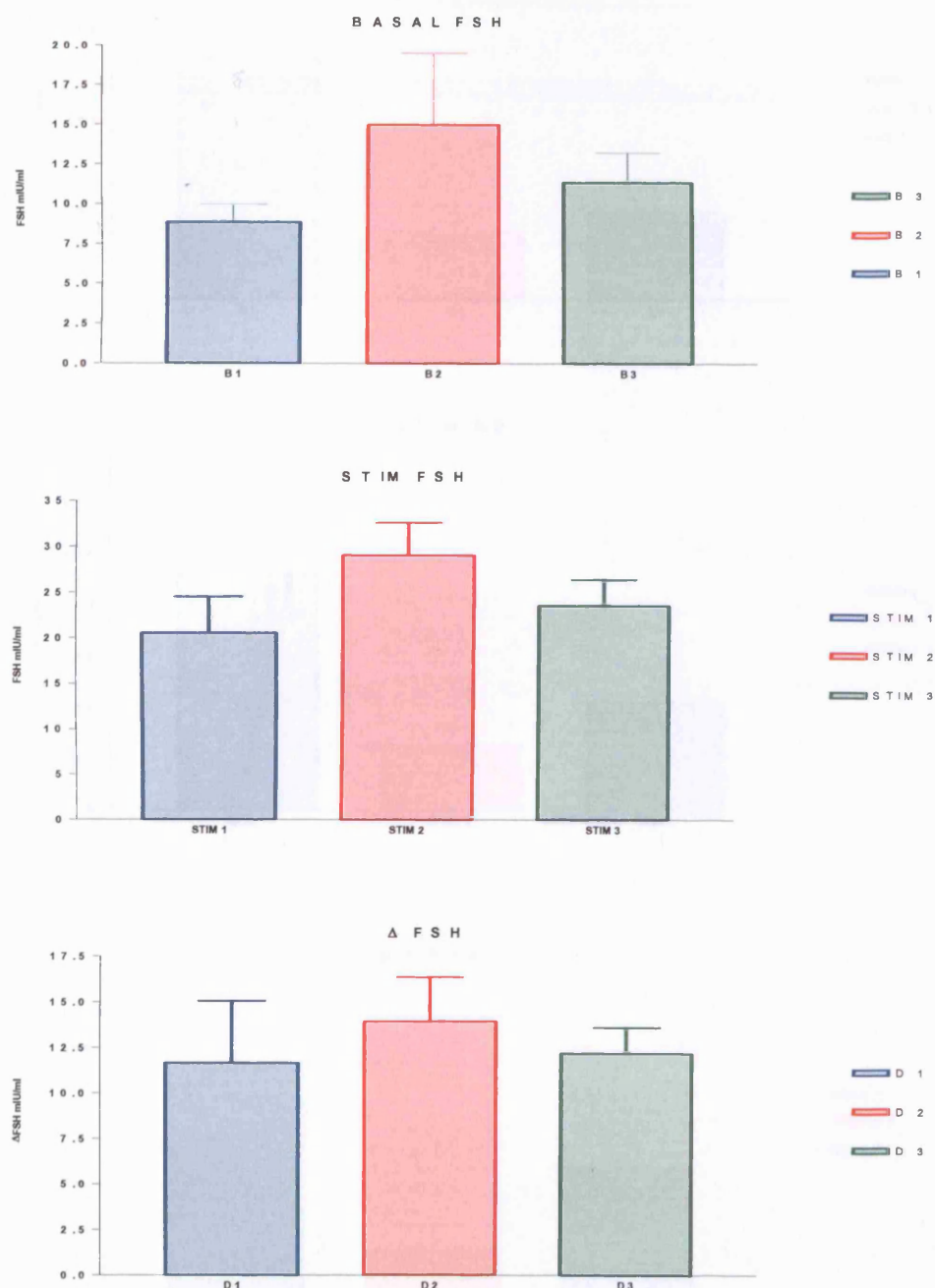


FIGURE 4.7.2.1 Serial FSH levels in 5 patients tested post-chemotherapy. Tests were performed at three consecutive 3-monthly time intervals.

4.7.2.2 OESTRADIOL

Basal and stimulated E2 levels were not significantly different between groups, however delta oestradiol was significantly different, overall $p = 0.02$.

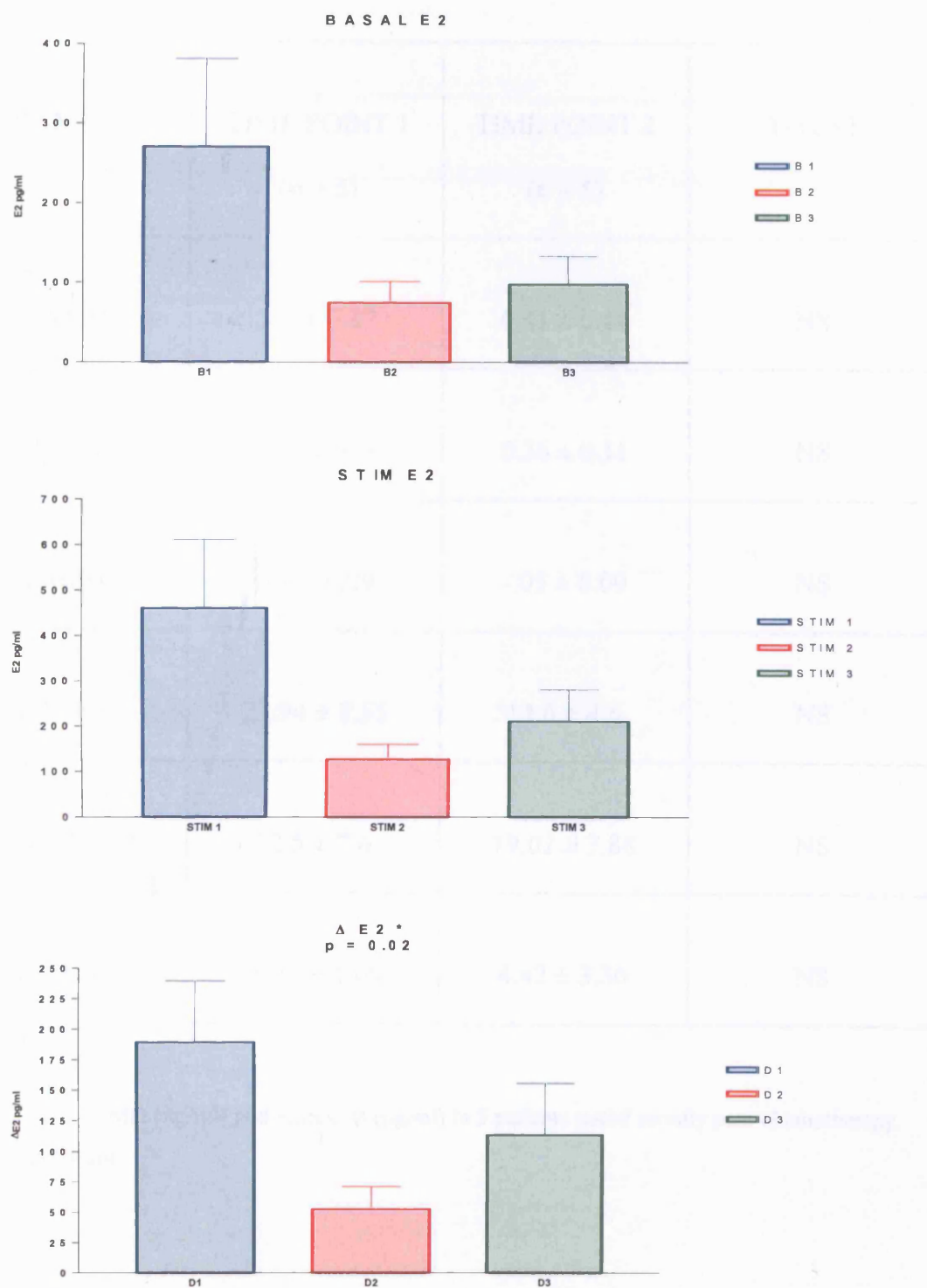


FIGURE 4.7.2.2 Oestradiol levels at 3 different time points.

$\Delta E2 p = 0.02$

4.7.2.3 AMH AND INHIBIN B

For these parameters, only two time points were available for analysis as there were insufficient numbers (n) for the third time point. No statistically significant difference was found.

PARAMETER	TIME POINT 1 (n = 5)	TIME POINT 2 (n = 5)	T-TEST
B AMH	1.9 ± 0.87	0.41 ± 0.14	NS
STIM AMH	2.01 ± 0.86	0.36 ± 0.11	NS
Δ AMH	0.1 ± 0.29	-0.05 ± 0.09	NS
B INH B	23.94 ± 8.55	14.6 ± 4.6	NS
STIM INH B	22.5 ± 7.6	19.02 ± 7.88	NS
Δ INH B	-1.44 ± 1.09	4.42 ± 3.36	NS

TABLE 4.7.2 AMH (ng/ml) and inhibin B (pg/ml) in 5 patients tested serially post-chemotherapy.

NS = not significant

4.7.3 BIOPHYSICAL PARAMETERS

4.7.3.1 OVARIAN VOLUME

Mean ovarian volumes (MOV) were not significantly different between the three time points ($p = 0.87$, $n = 4$).

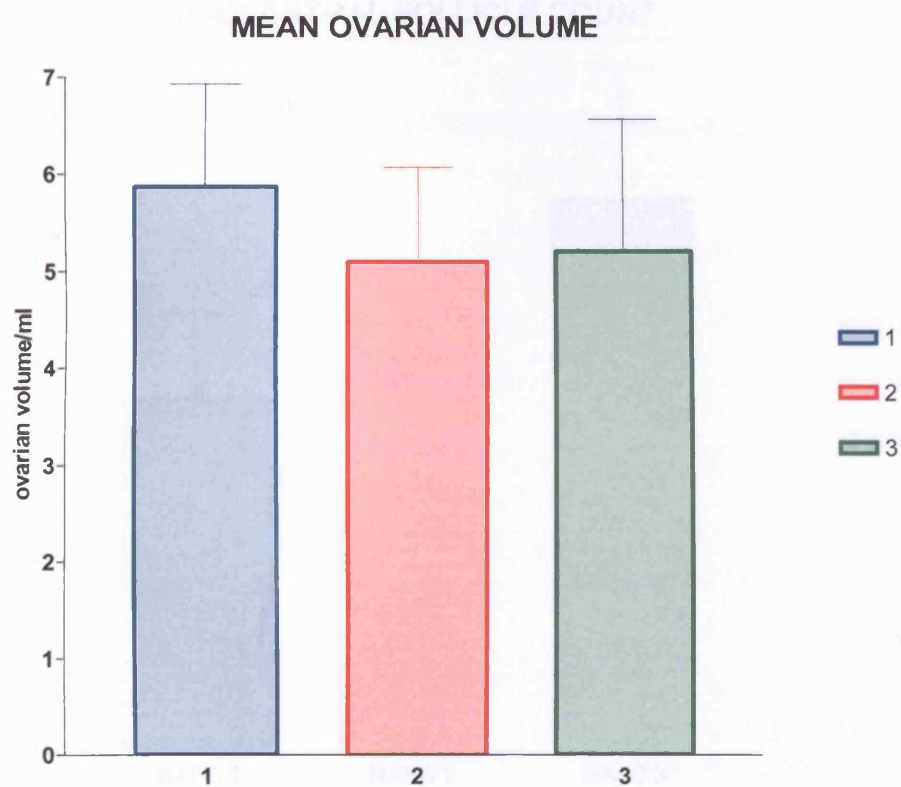


FIGURE 4.7.3.1 Mean ovarian volume at three different time points.

Although the overall $p = 0.05$, there was no significant difference between individual time points using Bonferroni's multiple comparison tests.

4.7.3.2 ANTRAL FOLLICLE COUNT

Overall there were no significant differences in the mean values for MAFC, although the p value was approaching significance ($p = 0.06$, $n = 4$).

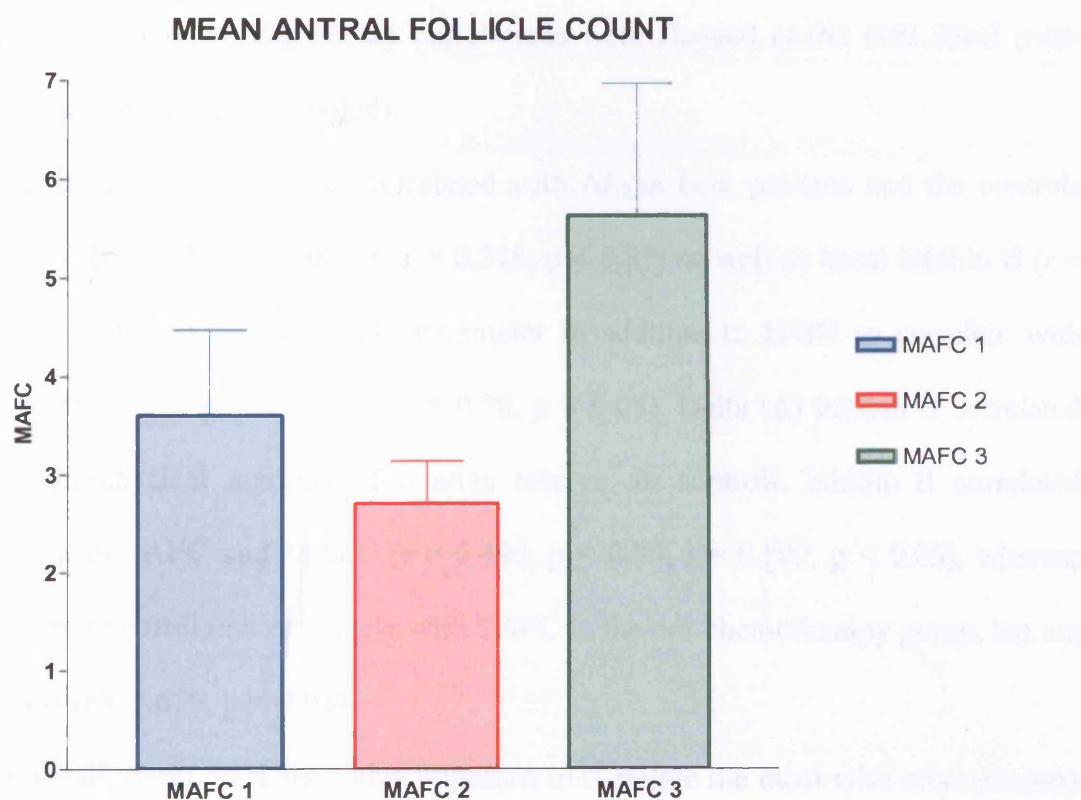


FIGURE 4.7.3.2 MAFC at three different time intervals in the same patients.

Overall $p > 0.05$.

4.8 CORRELATIONS

Correlation statistics were performed using SPSS comparing each of the ovarian reserve markers within each of the three groups studied: controls, pre-chemotherapy and post-chemotherapy patients respectively. Bivariate analysis was performed using Pearson's correlation coefficients. Significant correlations were flagged at the 0.01 level (two-tailed) or the 0.05 level (two-tailed).

AMH as a basal marker correlated with ΔE_2 in both patients and the controls respectively. ($r = 0.759$, $p < 0.001$; $r = 0.518$, $p < 0.05$) as well as basal inhibin B ($r = 0.842$, $p < 0.001$) It was the only parameter in addition to bFSH to correlate with chronological age in patients only ($r = 0.78$, $p < 0.05$). Delta (Δ) inhibin B correlated best with biophysical markers of ovarian reserve. In controls, inhibin B correlated positively with TAFC and MAFC ($r = 0.494$, $p < 0.05$; $r = 0.509$, $p < 0.05$), whereas delta oestradiol correlated positively with TAFC in the pre-chemotherapy group, but not the controls ($r = 0.670$, $p < 0.05$).

Overall, basal markers which appeared to correlate the most with other markers were AMH and inhibin B. Post-stimulation however, oestradiol and inhibin B levels correlated with both biochemical markers, including basal AMH, and were the only markers to correlate with AFC. No correlations with ovarian volume were identified.

4.9 LOST SAMPLES

An attempt was made to determine whether or not the samples affected by the freezer mishap could be utilized. This was possible because some of these samples had been assayed prior to the accident, thus allowing a comparison to be made between the results of assays performed on the same sample before and after the freezer mishap. Assays were repeated for oestradiol, inhibin B and AMH. Although the steroid assay (oestradiol) results appeared almost comparable, the results for inhibin B and AMH were too disparate. The decision was therefore made not to use any of the samples previously stored in the freezer.

OESTRADIOL:

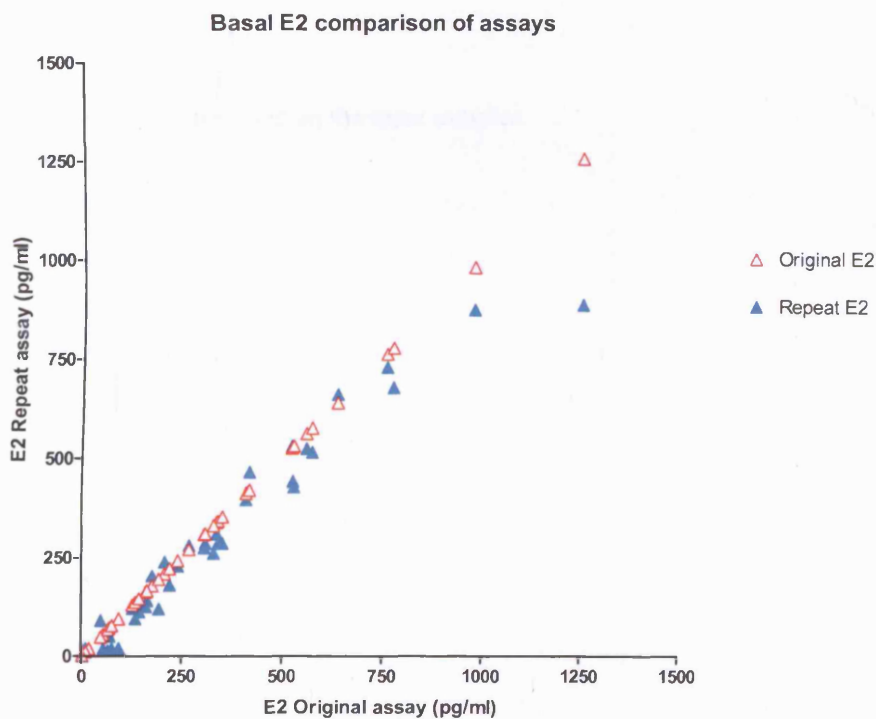


FIGURE 4.9.1 Oestradiol assay repeated on the same samples.

AMH:

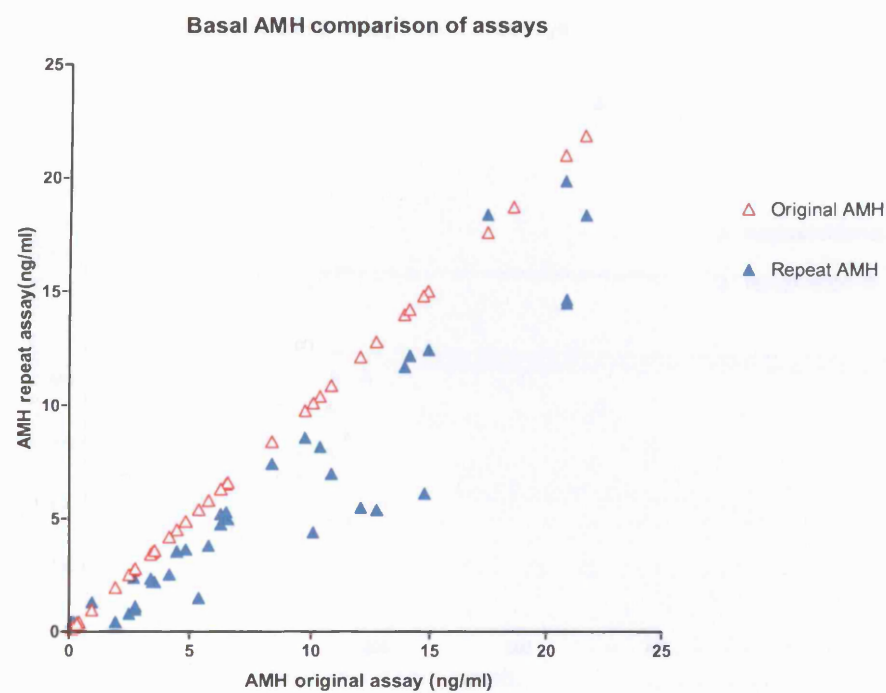


FIGURE 4.9.2 AMH assay repeated on the same samples.

INHIBIN B:

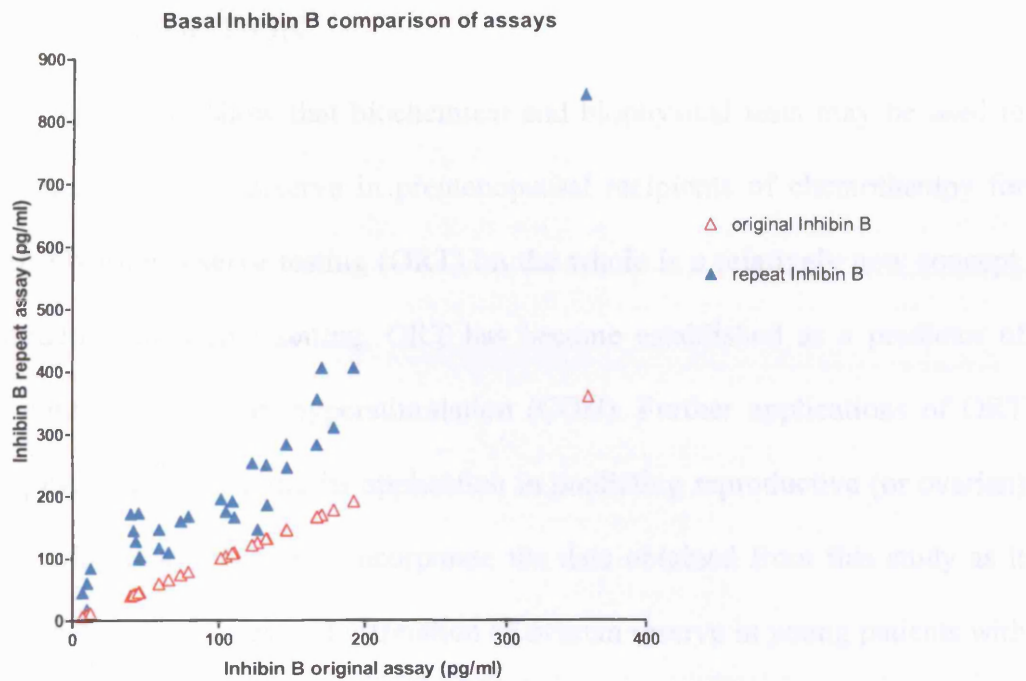


FIGURE 4.9.3 Basal inhibin B repeated in the same samples.

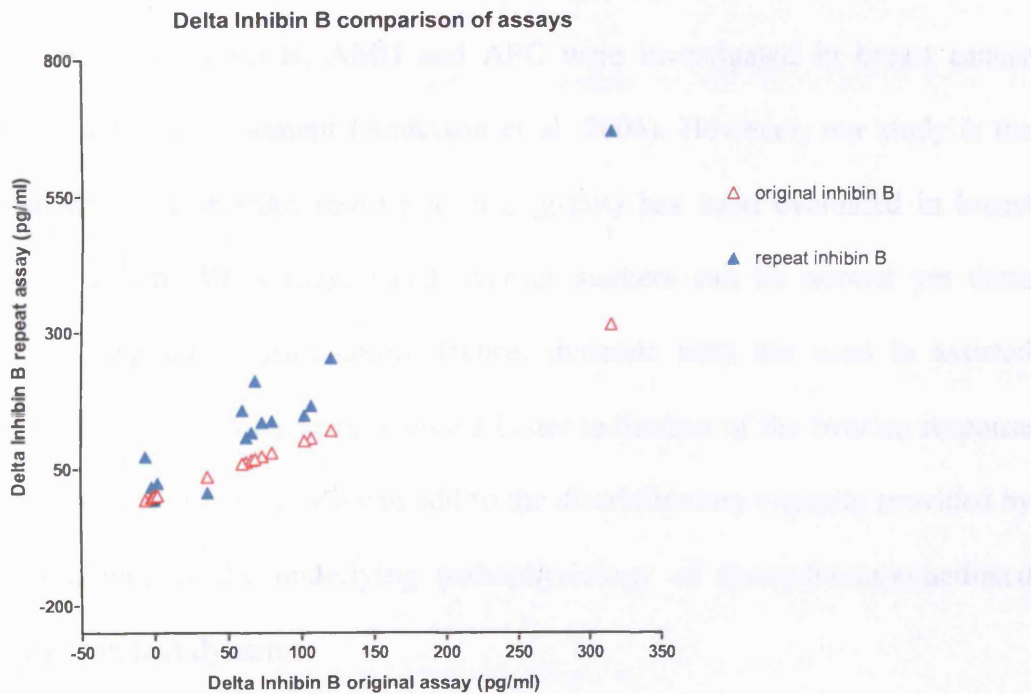


FIGURE 4.9.4 Delta inhibin B repeated in the same samples.

4.10 DISCUSSION

4.10.1 INTRODUCTION

The results of this study show that biochemical and biophysical tests may be used to accurately estimate ovarian reserve in premenopausal recipients of chemotherapy for breast cancer. Ovarian reserve testing (ORT) on the whole is a relatively new concept. In the reproductive medicine setting, ORT has become established as a predictor of response to controlled ovarian hyperstimulation (COH). Further applications of ORT have been investigated, including its application in predicting reproductive (or ovarian) age. The following discussion will incorporate the data obtained from this study as it pertains to the pathophysiology and estimation of ovarian reserve in young patients with breast cancer.

4.10.2 CROSS-SECTIONAL DATA

This study confirms the observation of a recent paper where the new ovarian reserve tests such as inhibin B, AMH and AFC were investigated in breast cancer patients before and after treatment (Anderson et al. 2006). However, our study is the first in which dynamic ovarian reserve testing (g-test) has been evaluated in breast cancer patients. In an IVF setting, basal ovarian markers can be normal yet these patients do not respond to stimulation. Hence, dynamic tests are used in assisted reproductive technology setting as they give a better indication of the ovarian response to stimulation. We believe the g-test can add to the discriminatory capacity provided by basal markers alone, as the underlying pathophysiology of chemotherapy-mediated gonadotoxicity is in fact dynamic.

In the recently published study, AMH concentration in particular was found to be useful as an early indicator of ovarian ageing, including the assessment of chemotherapy-induced ovarian follicle loss (Anderson et al. 2006). Contrary to this

study where the patients were 28-52 years of age, our study cohort comprised a significantly younger patient cohort (mean age of patients was 41.3 in the chemotherapy arm of the Anderson study, compared with 35.2 and 36.7 in the pre- and post-chemotherapy arms of our study – see figure 4.4.1.1), and included an age-matched control group with proven fertility. This was in keeping with our objective, which was to evaluate ORT in young women with breast cancer. It is this group where validation of ORT could potentially lead to adequate counselling and treatment with regards fertility preservation, at a time when therapeutic intervention is still feasible.

Previous studies in breast cancer focused mainly on the incidence of amenorrhoea and premature menopause respectively (Bines, Oleske, & Cobleigh 1996; Goodwin et al. 1999). Other investigators assessed the endocrine consequences of chemotherapy in patients with breast cancer, but the analysis was limited to gonadotrophin and steroid alterations respectively (Dowsett & Richner 1991; Mehta, Beattie, & Das Gupta 1992; Padmanabhan et al. 1987). These studies were performed long before the concept of ovarian reserve was realised. The importance of these alterations were accurately surmised as being detrimental to fertility, but the limitations of these studies prevented meaningful application in clinical practice (Hensley & Reichman 1998).

There are several reasons for the difficulty in assessing these patients appropriately. One of the factors involved includes the possibility of ovarian reserve being diminished even prior to chemotherapy being administered. A prospective study of ovarian function in patients undergoing bone marrow transplantation (BMT) for haematological malignancies identified evidence of gonadal insufficiency before BMT, which may have been exacerbated by conditioning regimens (chemotherapy and total body irradiation) prior to BMT being performed (Chatterjee et al. 1994). This was

evidenced by the significantly lower levels of oestradiol levels seen in patients following the HMG stimulation test compared with the controls.

Our data suggest that ovarian reserve was intact prior to chemotherapy in a breast cancer cohort, implying that multi-agent chemotherapy was the main factor that led to diminished ovarian reserve. Alkylating agents such as cyclophosphamide, which are non cell-cycle specific are more toxic to the ovaries than cell-cycle specific agents such as methotrexate and fluorouracil. It is unclear however, what effect multi-agent chemotherapy has on ovarian reserve, especially given the fact that newer agents are continually being developed (Awada et al. 2003). All the biochemical and biophysical markers with the exception of basal oestradiol (bE2) levels provided evidence that ovarian reserve was intact prior to chemotherapy (see section 4.4: cross-sectional data).

The G-test was chosen in this study as it provided an ovarian response, which was less potent than the HMG test, an important factor to consider in patients with breast cancer. While bE2 levels were significantly higher in patients (pre-chemotherapy) compared to the controls, there was no significant difference between stimulated and delta E2 levels. This may be explained by the fact that bE2 levels correlate more with the underlying disease, as it has been shown that high plasma oestradiol concentrations have been linked to breast cancer development regardless of menopausal status (Clamp, Danson, & Clemons 2002). Oestradiol increase in response to the G-test however, probably correlates better with ovarian reserve, which would explain the lack of a significant difference in patients compared with the controls in addition to the other markers of ovarian reserve (see figure 4.4.2.9).

At the mid-point of recruitment, our data also suggested at the time that basal activin A levels were higher in patients compared to the controls. This seemed plausible at the time, as elevated serum activin A levels had been detected in post-menopausal women with breast cancer (Reis et al. 2002). When the complete dataset was analysed

however, the mean activin A levels in patients pre-chemotherapy were not significantly different from the controls (see section 4.4.2.7). It would be necessary to analyse a greater cohort of patients and controls to elucidate the possibility that activin A levels are increased in premenopausal women with breast cancer.

Perhaps the main factor involved in assessing these patients appropriately is the fact that the pathophysiological process is dynamic, with both acute and cumulative effects. As such, efforts to grade the extent of ovarian damage, and indeed ovarian recovery by clinical, biochemical and biophysical parameters are limited by the dynamic processes involved (Chatterjee & Kottaridis 2002). Furthermore, extrapolation of data from one cancer cohort to another is inappropriate due to differences in disease, type of chemotherapy and the demographics of the population studied. A consideration for future study would be to perform ovarian reserve testing at different time points during the chemotherapy course. This might allow the acute nature of ovarian injury to be examined as part of the overall dynamic process.

In the fertility setting, the most important aspect of diminished ovarian reserve and the associated decline in reproductive potential is that its onset is highly variable (Scott, Jr. & Hofmann 1995). These points, in addition to the fact that there is a paucity of prospective, controlled data outside a reproductive medicine setting, makes current evaluation of markers of ovarian reserve very difficult indeed. To the best of our knowledge, it appears that no single marker can be clinically useful in assessing ovarian reserve, leading to the evaluation of multiple markers (Lutchman Singh, Davies, & Chatterjee 2005).

These recognised limitations of studying ovarian reserve in patients with cancer greatly influenced the study design employed. For example, a single cancer cohort was studied as opposed to variable cancer types. Patients and controls were recruited only if they had regular menstrual cycles (26 to 34 days). Furthermore, it was anticipated that

young patients with breast cancer were likely to have high grade, oestrogen receptor (ER) negative disease, and were likely to receive adjuvant chemotherapy. Controls were chosen who were healthy, age-matched and had proven fertility by virtue of having at least one childbirth in the past. Although this made recruitment of controls more difficult, it helped to ensure that the results obtained were more likely to represent a gold standard of reference ranges for ovarian reserve in healthy controls than other examples quoted in the literature. Nevertheless the mean and median values for biochemical and biophysical parameters of ovarian reserve for the controls in this study compare favourably with control data reported elsewhere (Fitzgerald et al. 1994; Macnaughton et al. 1992; Muttukrishna et al. 2000; Ng et al. 2003; Pavlik et al. 2000; Scheffer et al. 1999; Welt et al. 1999). There is a distinct paucity in the literature however regarding reference ranges for newer markers of ovarian reserve such as AMH.

Clinical characteristics alone are not useful in predicting ovarian reserve (hence the original motivation behind the identification of other markers). However, significant alterations in clinical characteristics can alter the interpretation of results. Hence it is important to point out that in this study; there were no significant differences in age, body mass index (BMI) or cycle length (25 to 34 days) between patients and the controls.

Biochemical parameters, which appeared to discriminate between patients and the controls in the cross-sectional dataset, were serum FSH, oestradiol, inhibin B and AMH. The data relating to the use of FSH in estimating ovarian reserve in breast cancer is limited. Though widely used in a reproductive medicine setting, its main limitations arise from a lack of reproducibility (Sharara, Scott, Jr., & Seifer 1998). The pulsatile nature of FSH secretion is believed to be one of the factors responsible for this variation. To minimise this possibility, we took four (4) timed samples, 15 minutes apart and used a mean value as a “true” representative figure. Basal FSH levels were significantly

different across the three groups (controls, pre-chemotherapy and post-chemotherapy patients, $p = 0.0006$ – see figure 5.4.1). Following the G-test, stimulated FSH levels were also significantly different ($p = 0.01$ – figure 4.4.2.2). However, delta FSH levels did not confer any discriminatory capacity (figure 4.4.2.3). FSH is an indirect marker of ovarian reserve and depends on the presence of an intact hypothalamic-pituitary-ovarian (HPO) axis. Administration of the G-test causes a supraphysiologic increase in FSH causing a temporary increase in pituitary secretion of FSH and LH, to which the ovaries respond by releasing oestradiol. It is the amount of oestradiol released (delta) which correlates with ovarian reserve, not delta FSH, which is in keeping with the results. This overall increase in oestradiol production by the developing follicles would eventually suppress FSH levels, thus forming the basis of the CCCT, which was not used in this study.

Basal oestradiol (E2) levels were significantly higher in the pre-chemotherapy group compared to the controls. This may have a role in the pathophysiological process of breast cancer as described earlier. No significant difference existed between the control and the post-chemotherapy group. In fact, higher levels of basal E2 were seen in the pre-chemotherapy group as opposed to the post-, which one would not expect, as higher levels of E2 are associated with diminished ovarian reserve. Both stimulated and delta E2 levels were significantly different however, being much lower in the post-chemotherapy group compared with the pre-chemotherapy group and the controls respectively. This supports the notion that the G-test confers discriminatory capacity.

If delta E2 were significantly different because of the G-test, then one would expect inhibin B levels to be significantly different as well, due to the regulatory role inhibin B has on FSH secretion (specifically monotropic FSH rise). Not surprisingly therefore, basal, stimulated and delta inhibin B levels were all significantly different between patients and the controls (figures 5.4.2.13, 5.4.2.14 and 5.4.2.15). Whilst

inhibin B is mainly secreted by pre-antral follicles (Klein et al. 1996), inhibin A is produced primarily during the late follicular phase by mature follicles and the corpus luteum (Roberts et al. 1993). This would explain why the former is a more reliable marker of ovarian reserve, as our results confirm. The added discriminatory capacity as provided by the G-test for inhibin B and oestradiol is consistent with the theory that the pathophysiological processes taking place within the ovary are in fact dynamic – thus necessitating a dynamic form of assessment.

AMH is considered a direct marker of ovarian reserve as it is produced by FSH-sensitive early antral follicles. In this way, it may be a more sensitive predictor of ovarian reserve than other markers such as AFC and inhibin B, which detect more mature primordial follicles. AMH expression by granulosa cells is relatively stable (Gruijters et al. 2003), and is independent of GnRH or gonadotrophin regulation, with a relatively stable expression over the menstrual cycle (Cook et al. 2000; La Marca et al. 2006). This would explain why serum AMH levels were mostly unchanged in our study following the G-test (figures 4.4.2.11 and 4.4.2.12), a phenomenon detected in other studies (Pastor et al. 2005; van Rooij et al. 2002). Our results confirm that basal AMH levels were dramatically reduced in the post-chemotherapy group compared to both the control and the pre-chemotherapy group (figure 4.4.2.10). This is in keeping with another study which assessed ovarian reserve in survivors of childhood cancer (Bath et al. 2003). In that study, inhibin B failed to discriminate between patients and the controls while FSH levels did. This is a surprising result, and is in contrast with our own data. Given the fact that inhibin B has a crucial role in regulating FSH response, it is difficult to explain the pathophysiology of that finding adequately. What is much more likely, as has been the problem with ovarian reserve studies in general, is that a methodological flaw was exposed.

All other parameters tested, including activin A, follistatin and pro α C were found not to be discriminatory. This mirrors the situation in assisted reproduction, where none of these markers have been found to be useful predictors of ovarian reserve.

With regard to biophysical parameters, our data suggests that AFC is most useful, with AFC being significantly reduced in patients post-chemotherapy compared to the controls (figure 4.4.3.3). AFC has been extensively studied recently in the reproductive medicine setting and there is no doubt that its popularity as a marker of ovarian reserve has gained considerable momentum. In a study which used a control group very similar to our own, AFC correlated best with chronological age, as well as stimulated E2 and inhibin B levels (Scheffer et al. 2003). One could argue that stimulated inhibin B levels reflect the response from a larger group of small, FSH sensitive follicles not yet detectable on USS, whereas E2 stimulation reflects larger, visible antral follicles capable of E2 production. If this is the case, it is debatable whether a stimulation test such as the G-test adds any discriminatory capacity to AFC alone. Several studies have been published in a fertility setting where AFC correlates well with the response to exogenous gonadotrophins (Bancsi et al. 2002; Hendriks et al. 2005). It would be useful therefore to examine AFC immediately after ovarian stimulation occurred. We attempted to do this in three patients and three controls (data not shown). Overall, there was no difference in AFC or any other biophysical parameter at the beginning or the end of the G-test respectively. This is in keeping with the observations of other investigators, where AFC had been assessed as part of the CCCT in patients undergoing IVF. In that study, no significant differences were detected between basal, stimulated and down-regulated AFC (Ng et al. 2005).

While studies in cancer are limited, AFC and ovarian volume have been found to be useful in assessing ovarian reserve in childhood survivors of cancer (Larsen et al. 2003a; Larsen et al. 2003b) and patients undergoing BMT (Chatterjee et al. 1994). Our

results did not show a significant difference in OV between patients and the controls (figure 4.4.3.1). There is still some debate as to the usefulness of OV in predicting ovarian reserve. While it is clear that ovarian volume measurements are known to be discriminatory in patients who are postmenopausal (Flaws et al. 2000; Flaws et al. 2001), its value in predicting reproductive age is unreliable. This may be because the sensitivity and specificity of the test in identifying non-pregnancy is reduced in patients who are still having regular menses, thus allowing more subtle alterations in ovarian reserve to be missed. If this was true, then OV would have limited application in assessing ovarian reserve in patients with cancer, as subtle alterations in ovarian reserve are paramount in identifying those at risk of a premature menopause. There remains the possibility that the imaging modality itself may be the limiting factor regarding the usefulness of OV in assessing ovarian reserve. The recent introduction of 3D USS into clinical practice might prove the solution for this. The results of further research into this area are anticipated.

The other biophysical parameters tested included uterine dimensions (uterine cross-sectional area and endometrial thickness) and ovarian stromal blood flow (PI and MPS). None of these parameters were significantly different between patients and the controls. In the case of uterine dimensions, one would probably not expect any significant changes unless pelvic or total body irradiation (TBI) were administered, as is often the case in haematological malignancy (Meirow & Nugent 2001). Ovarian stromal blood flow theoretically would be expected to be more useful. Studies however are still very limited. The most recent, from a reproductive medicine setting imply no use for OSBF to predict response to IVF using 2D power doppler (used in this study) (Ng et al. 2005) or even 3D doppler (Ng et al. 2006).

Correlations were performed to examine relationships between individual markers of ovarian reserve. It is interesting to note that only bFSH in the post-

chemotherapy group, and bAMH + bE2 in the pre-chemotherapy group correlated with chronological age. This is probably because the age range of the study subjects was quite narrow, preventing a meaningful correlation statistic. The median age of patients was 35.5 and 37 (pre- and post-chemo) years respectively thus necessitating a similar age ranges for the controls. As such, most of the study group were already in their mid-thirties, a time when reproductive ageing is known to accelerate (Baird et al. 2005). It was entirely appropriate to examine this age group however, as it the one most relevant to patients with breast cancer. The responses of oestradiol and inhibin B to the G-test correlated highly with bAMH and AFC respectively, but not with FSH. This adds to the hypothesis that these markers of ovarian reserve are inter-related, each possibly providing a different aspect of information on ovarian reserve. The right combination of these factors therefore may produce the most accurate assessment of ovarian reserve in these patients.

4.10.3 PROSPECTIVE DATA

While the cross-sectional data discussed earlier provided very useful insight into the evaluation of ovarian reserve markers in breast cancer, it is unfortunate that sufficient data was not accrued prospectively as it might have conferred some predictive capacity. The reasons for this will be explained when the methodology is discussed in more detail. Nevertheless, it was intuitive to observe the variation in markers of ovarian reserve over time in selected patients.

For most parameters in the longitudinal group, it was possible to obtain eight paired samples, representing pre-and post-chemotherapy samples respectively. As already mentioned, there were no significant differences between patients and the controls, with the exception of basal oestradiol. All longitudinal patients became

amenorrhoeic during the course of their chemotherapy. As such, only basal parameters were compared pre-and post-chemotherapy, since the G-test could not be performed.

The parameters, which were discriminatory between these two groups, were FSH, E2 and AFC. It can be deduced that the multi-agent chemotherapy received by these patients suppressed ovarian function resulting in a hypoestrogenic state, with a corresponding high FSH and amenorrhoea (figure 4.5.2.1). Reduced AFC probably reflects a reduction in the number of mature antral follicles capable of producing E2 (figure 5.5.2.3). Ovarian volume, as was the case in the cross-sectional data, was not significantly different between both groups (figure 4.5.3.1). This may be a reflection of the fact that the induced hypoestrogenic state was temporary. It can be argued that in terms of reproductive ageing, a reduction in ovarian volume is a relatively late event that comes about after a protracted period of diminished ovarian reserve. It is plausible that despite these patients being amenorrhoeic, the unchanged ovarian volumes implied that resumption of ovarian function, albeit at a level lower than the pre-chemotherapy state, was likely to occur. In fact, all patients in the post-chemotherapy arm of the cross-sectional analysis had resumed normal menses, indicating that the effects of the multi-agent chemotherapy received were at least partially reversible.

Basal AMH and inhibin B levels (figures 4.5.2.4 and 4.5.2.5) were not significantly different, as was the case with the cross-sectional data. This was likely because insufficient pairs of samples were analysed – three pairs compared to eight for the other parameters.

In three patients, it was possible to assess ovarian reserve parameters after the resumption of menses had occurred (section 4.6). It was possible to perform the G-test in these patients, where it was shown that the ovarian response to gonadotrophins was well below that compared to the pre-chemotherapy state. While meaningful statistical comparisons were not possible, the data clearly showed resumption of ovarian activity

(biochemical and biophysical) at a variable level from the pre-chemotherapy state. For example basal FSH showed a distinct rise immediately following chemotherapy but appeared to fall over the following 6 months, a trend which was also apparent following the G-test (figure 4.6.2.1). Basal oestradiol levels were reduced following chemotherapy, but it was interesting to note that delta oestradiol levels remained low even up to six months after resumption of menses (figure 4.6.2.2). This is in keeping with the hypothesis that the pathophysiological processes involved are in fact dynamic and variable, with delta oestradiol potentially providing additional discriminatory capacity in terms of ovarian reserve. In terms of biophysical parameters, AFC appeared to show a trend of marked reduction immediately following chemotherapy, with delayed recovery up to six months after resumption of menses (figure 4.6.3.2).

These conclusions are based on a small sample size. To properly elucidate the course of ovarian recovery, further longitudinal follow up with a larger number of patients would be necessary to allow stratification according to chemotherapy type and dose.

Prospective data was also accumulated on five patients who were recruited after completion of chemotherapy (section 4.7). In these patients, it was possible to perform dynamic testing on at least 3 occasions. There was a significant difference overall in delta E2 between the three time points tested. There was not a gradual increase or decline however, but a fluctuating pattern, despite the fact that the mean time from completion of chemotherapy to the time of testing was 34 months. This suggests that although ovarian function is present, reduced ovarian reserve results in variation of ovarian function. Of course this possibility can only be adequately tested with a larger number of patients followed up to a fixed outcome – the ideal one being onset of premature menopause.

4.10.4 METHODOLOGICAL DIFFICULTIES

The aim of this study was to evaluate biochemical and biophysical markers of ovarian reserve in young patients with breast cancer. Being the first study of its kind in a breast cancer cohort, the study methodology designed in a robust fashion in order to ensure that reliable data was obtained, which would eventually form the basis of a larger study.

Recruitment of patients was difficult. Although the incidence of breast cancer is rising in the UK, it is still an uncommon disease in women aged less than 40 years and even more so in women aged less than 35 years. Suitable candidates for the study were first identified at their first discussion at the breast MDT meeting. At that point patients have either had primary surgery or been considered for primary chemotherapy. There is limited time from that point before the commencement of chemotherapy. Patients are then seen by the medical oncologist and treatment usually commenced within two weeks after that visit. At a time when patients are confronted with the enormous task of digesting their diagnosis and treatment plan, there was very little time to discuss the study with them and obtain informed consent. Despite this, most patients who were approached shared a great interest in taking part in the study.

Furthermore, all tests were well tolerated with no short-term adverse effects reported.

The main obstacle perhaps was to perform the ovarian reserve testing during the early follicular phase. Treatment was initiated regardless of their menstrual cycle status. If patients were recruited after the early follicular phase had passed, it was considered unethical to ask them to wait until their next menstrual cycle before starting chemotherapy. Interestingly, a recent study has shown the incidence of amenorrhoea following chemotherapy in women with early breast cancer was actually increased if treatment was started in the follicular phase (Di Cosimo et al. 2004).

Patients requesting fertility preservation were also recruited. Embryo or oocyte cryopreservation were the options commonly available for these patients. In both cases, a controlled ovarian hyperstimulation is required to allow harvest of oocytes. This involved either long stimulation protocols (initial downregulation in the luteal phase followed by hyperstimulation), or if time was limited, short GnRH agonist protocols without conventional downregulation. This again made it very difficult to perform ORT at the specified time. It would be possible to assess ovarian reserve in these patients by virtue of their response to COH however, a valid consideration for future study as this group has the advantage of defined end-points for study (oocytes retrieved) allowing short-term predictive value to be potentially established.

Biophysical tests were performed mostly by a single operator (Ms Anita Patel) using a combination of transvaginal and abdominal USS. On a few occasions, it was necessary to have the biophysical profile performed by another experienced ultrasonographer (Dr B Parikh). Some of the observations were repeated by Ms Patel to ensure that inter-observer variability was minimized.

Biochemical assays were done under the supervision of Dr Muttukrishna. Well-established in-house assays were performed when possible, otherwise commercial kits were used. After a six-month laboratory and ELISA training process, it was then possible for me to carry out all assays under indirect supervision. A rigid documentation process was utilized to allow rapid review of errors and to ensure reproducibility.

The most important difficulty faced during this study occurred during sample storage. All serum samples were centrifuged within two hours of venesection and the serum stored in quadruplicate at -20°C until ready for assaying. Some of these samples were assayed at the midpoint of the study to obtain preliminary data. As per usual laboratory protocol, samples were performed in batches to reduce inter and intra-assay variability. Unfortunately there was a complete failure of the freezer mechanism

(including the failsafe), which resulted in the samples being left at room temperature for an unknown time during a Christmas vacation period. There was no precedent in the literature to guide what use was possible with these samples. We decided to re-assay the samples that were done previously to compare the results (section 4.9). This revealed that although the gonadotrophin and steroid results appeared similar, the protein-based hormones such as AMH and inhibin showed large discrepancies. There was no option but to destroy these “spoiled” samples without using the data. This incident caused the loss of many longitudinal samples thus severely affecting the prospective arm of the study. Where possible, volunteers were asked to have their tests repeated. Further samples were then stored in quadruplicate, however 2 samples each were placed into a -80°C and -20°C freezers respectively.

4.10.5 SUMMARY

This study confirms the use of biochemical and biophysical parameters of ovarian reserve in a breast cancer cohort, and is the first to introduce the concept of dynamic ovarian reserve testing in this group. The methodology was robust and included elaborate methods of testing – all of which appeared to be well tolerated. The cohort was disease standardised and received similar types of treatment which were cyclophosphamide based. Biochemical markers, which appeared to be discriminatory, included FSH, AMH, inhibin B and oestradiol in response to the G-test. The only clearly discriminatory biophysical marker was the antral follicle count. There was good correlation between AFC and other markers of ovarian reserve. These results taken together support the hypothesis that ovarian reserve is reduced in young, regularly cycling women following treatment with chemotherapeutic agents for breast cancer. The data also add to the understanding of the pathophysiological processes involved.

These findings have major implications for breast cancer survivors, for whom reproductive issues are a major concern (Partridge et al. 2004). Furthermore, the potential exists for ovarian reserve testing to be applied in patients with different types of cancer.

To achieve this, a large sample size should be followed up longitudinally to determine the potential therapeutic role of ovarian reserve testing in this cohort.

5. RESULTS IN VITRO STUDY

5.1 INTRODUCTION

The background, aim and study design for the in vitro study have already been discussed in detail in chapters 3 and 4.

BREAST CELL CULTURE:

Briefly, MCF-7 cells were maintained in Dulbecco's Modified Eagle Medium plus Ham's F12 (DMEM/F12) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. The cells were plated in 24-well plates at 20,000 cells per well and incubated overnight. A dose response for each drug was then obtained by applying standard dilutions of each drug to the plate in quadruplicate and monitoring its effect using an inverted phase contrast microscope. Once the dose range for each drug was established, experiments were carried out a minimum of six times, with each plate having a 72-hour exposure of each drug. Following this exposure, the cells were lifted with 0.02% Trypsin and EDTA, washed with washed with phosphate buffered saline (PBS) and resuspended in Annexin-V-Fluos/ Propidium Iodide solution prior to analysis in a Flow Cytometer. The medium from each well was also centrifuged and the resulting pellet of "dead" cells washed and resuspended as above to add to the flow cytometric analysis.

GRANULOSA CELL CULTURE:

Briefly, cells obtained from different follicles in the same patient were pooled and centrifuged at 1100 rpm for 10 minutes. The supernatant was discarded and the cells washed and resuspended with Hanks balanced salt solution (HBSS) containing 1% penicillin/streptomycin. The granulosa cells were separated from red blood cells on a

60% Percoll layer, washed with HBSS and the resulting cell aggregate incubated for 15 minutes with 0.1% bovine testicular hyaluronidase in 2ml HBSS. After washing with HBSS the cells were mechanically dispersed, washed in HBSS and resuspended in DMEM/F12 supplemented with 1% penicillin/streptomycin, 1% amphoterecin, 0.1% gentamycin and 20% FCS. Cell viability was determined by the trypan blue exclusion method and cells counted using a hemocytometer. Cells were plated at a density of 20000 cells per well and incubated overnight in a water saturated incubator with 5% CO₂ at 37° C for drug exposure the following day. Administration of drug was identical to that described for the breast cells with an exposure of 48 hours. A shorter exposure time was used for the granulosa cells as it was difficult to maintain the viability of the cells for longer periods. As described earlier, cells were lifted with EDTA/Trypsin and cells from quadruplicate wells were pooled for flow cytometry. The medium from each well was stored separately, and later pooled for assays of inhibin A and oestradiol.

5.1.1 DATA ANALYSIS

Using annexin/PI staining as described previously, each cell culture experiment was quantified into 4 domains (D):

ANALYSIS PARAMETER	DEFINITION
D1	NECROTIC CELLS
D2	LATE APOPTOSIS
D3	LIVING CELLS
D4	EARLY APOPTOSIS

TABLE 5. 1 Interpretation of cytometric data.

Viable cells are quantified in D3. Early and late apoptosis characterizes dying and dead cells respectively (D2 + D4).

All parameters passed normality testing (Gaussian distribution). The data were then normalized to achieve maximum significance. Dose-response curves were obtained for a minimum of 6 experiments. From these experiments, one-way analysis of variance was used to obtain the mean viability or cell death for each drug concentration used. Log-transforming the drug concentrations and non-linear regression curves were then employed to obtain the LD₅₀. All Statistical analyses were performed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego California USA) and Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA).

5.2 CYTOMETRY RESULTS

5.2.1 BREAST CELLS

DOXORUBICIN

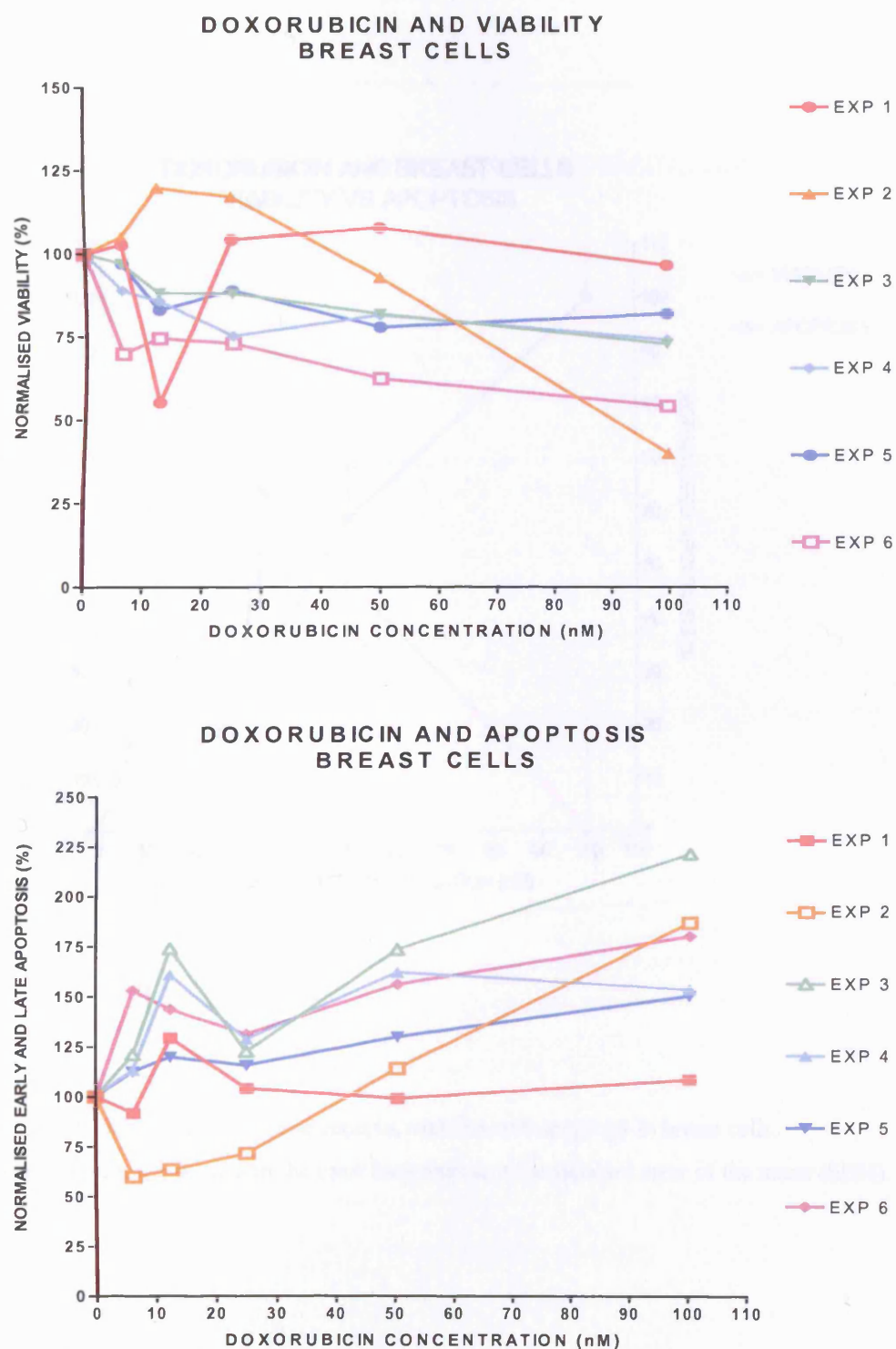


FIGURE 5.2.1.1 Effect of doxorubicin on cell viability and apoptosis in MCF-7 cells.

DOXORUBICIN AND BREAST CELLS – MEANS PLOTS:

The means plots were derived from using one-way ANOVA. The overall significance for the difference between groups was $p = 0.06$ for normalised apoptosis and $p = 0.05$ for normalised viability respectively ($n = 6$).

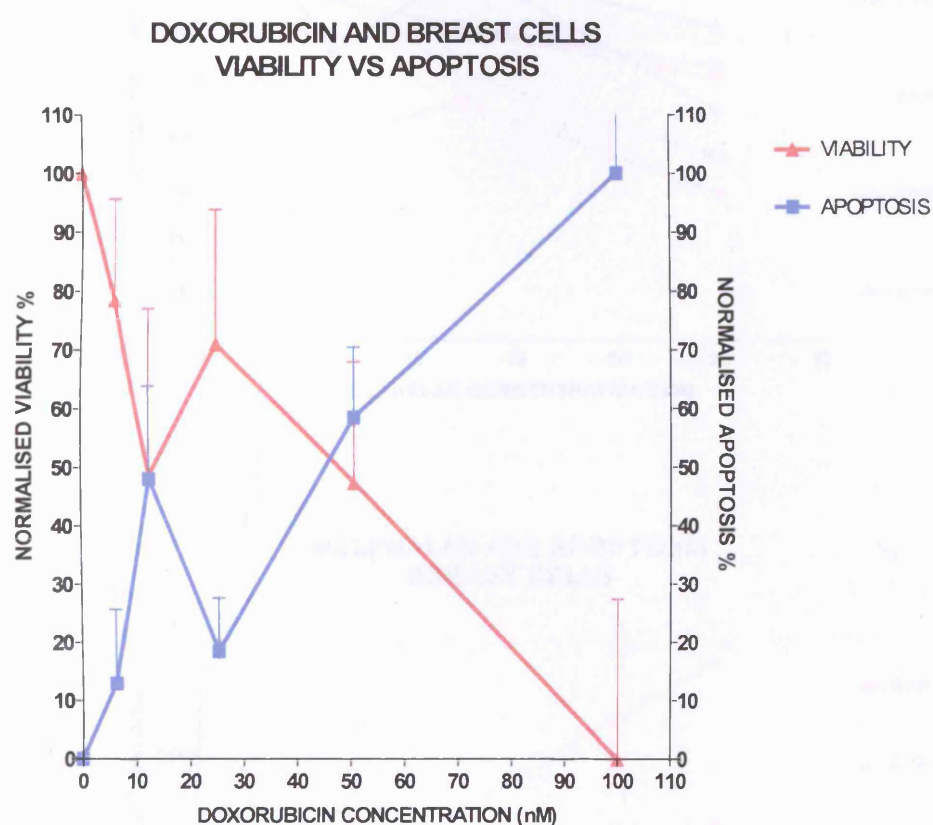


FIGURE 5.2.1.2 Doxorubicin concentration vs. viability and apoptosis in breast cells.

Data points represent the mean and the error bars represent the standard error of the mean (SEM).

MELPHALAN

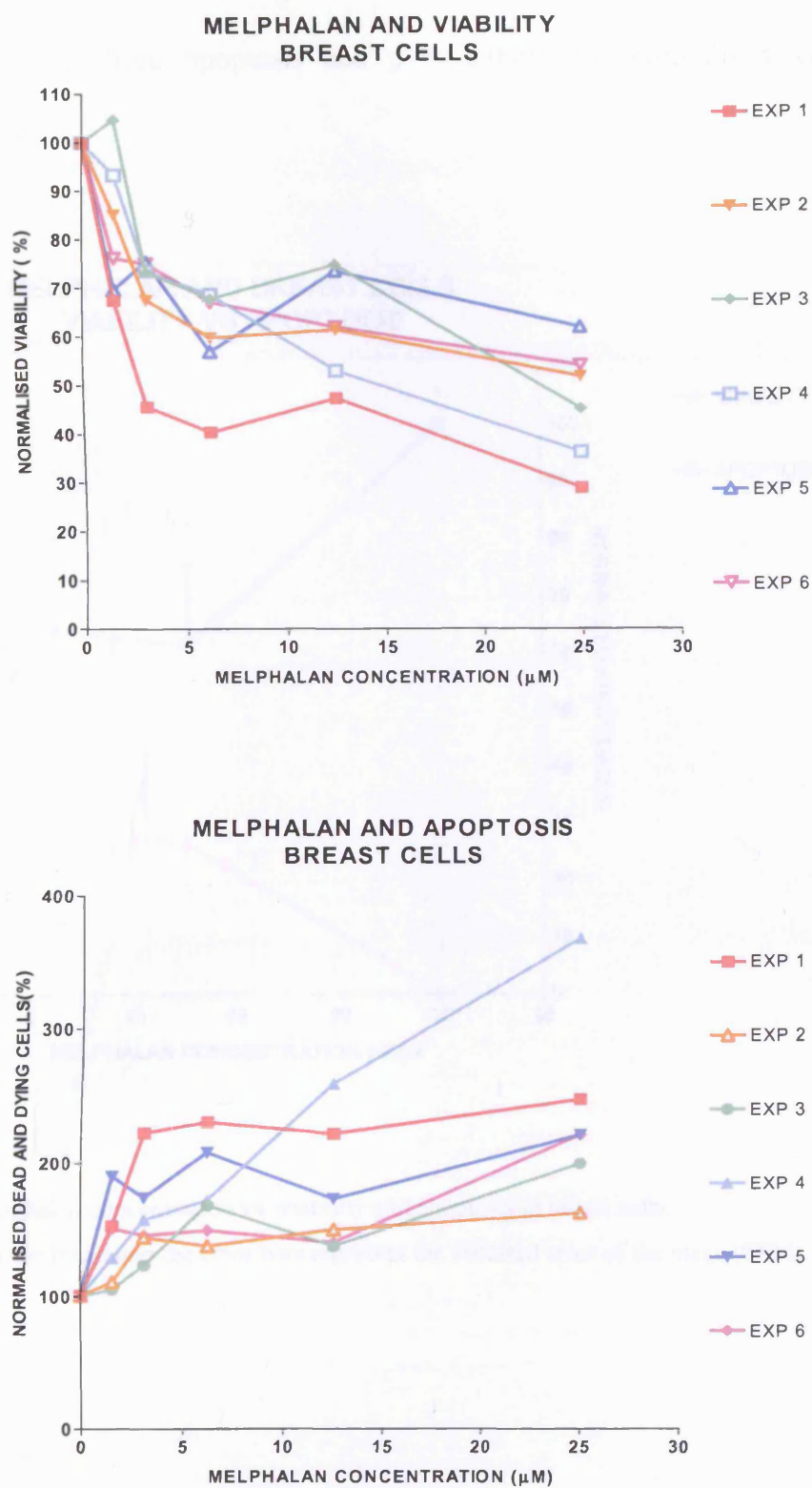


FIGURE 5.2.1.3 Effect of melphalan on cell viability and apoptosis in MCF-7 cells.

MELPHALAN AND BREAST CELLS - MEANS PLOTS:

Using one-way ANOVA, the overall significance for the difference between groups was $p = 0.0001$ for normalised apoptosis and $p = 0.0001$ for normalised viability respectively ($n = 6$).

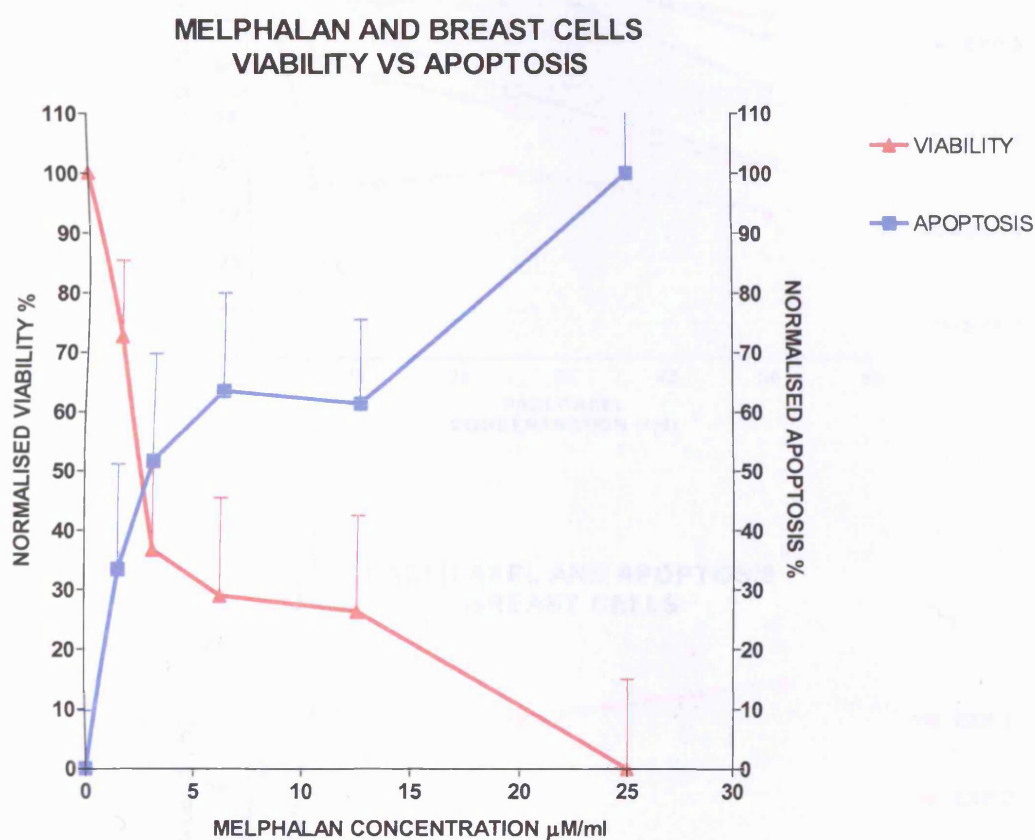


FIGURE 5.2.1.4 Melphalan concentration vs. viability and apoptosis in breast cells.

Data points represent the mean and the error bars represent the standard error of the mean (SEM).

PACLITAXEL

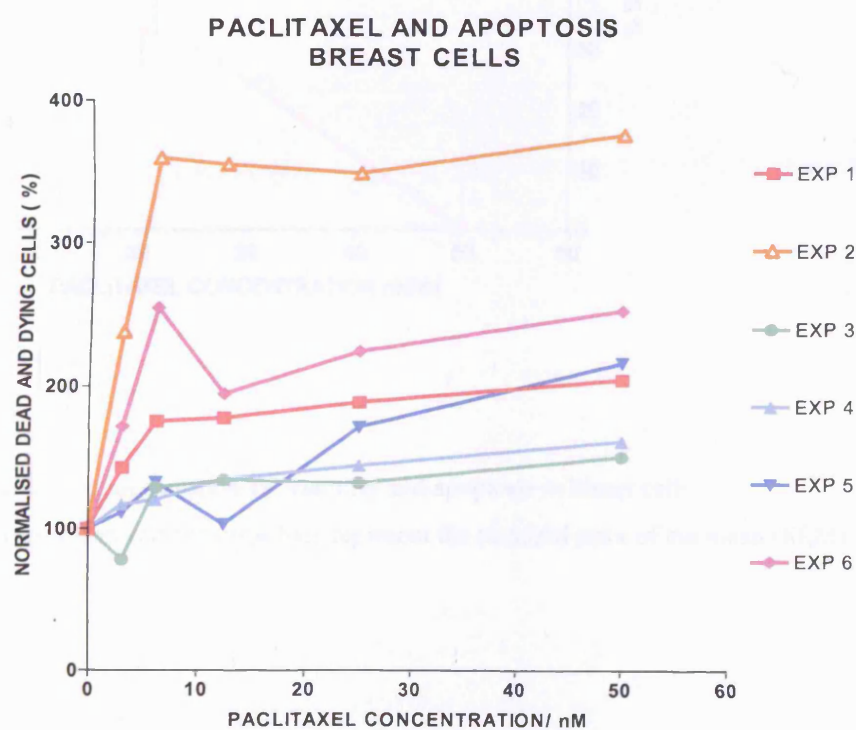
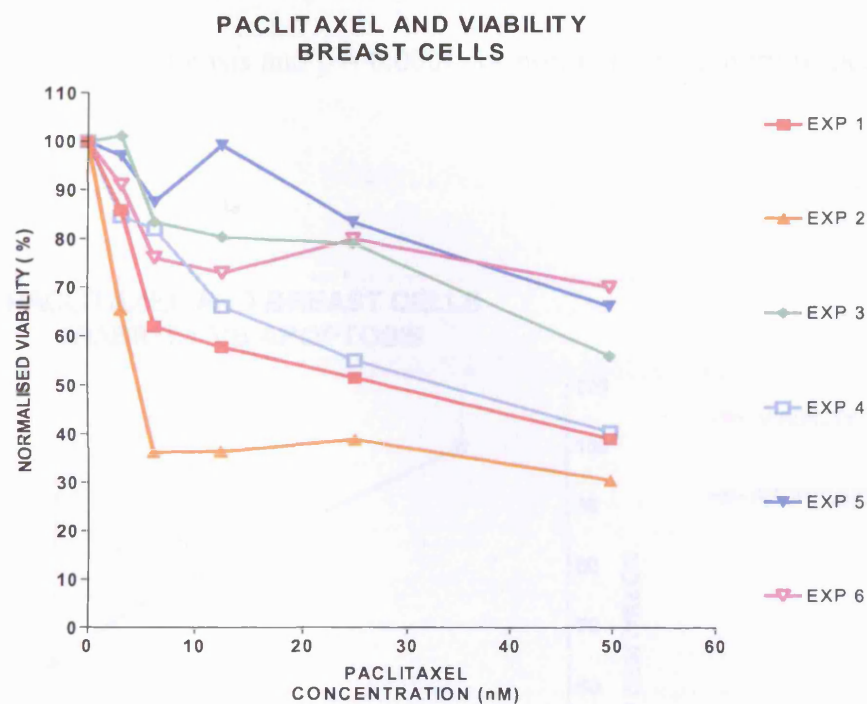


FIGURE 5.2.1.5 Effect of paclitaxel on cell viability and apoptosis in MCF-7 cells.

PACLITAXEL AND BREAST CELLS - MEANS PLOTS:

Using one-way ANOVA, the overall significance for the difference between groups was $p = 0.007$ for normalised apoptosis and $p = 0.0001$ for normalised viability respectively ($n = 6$).

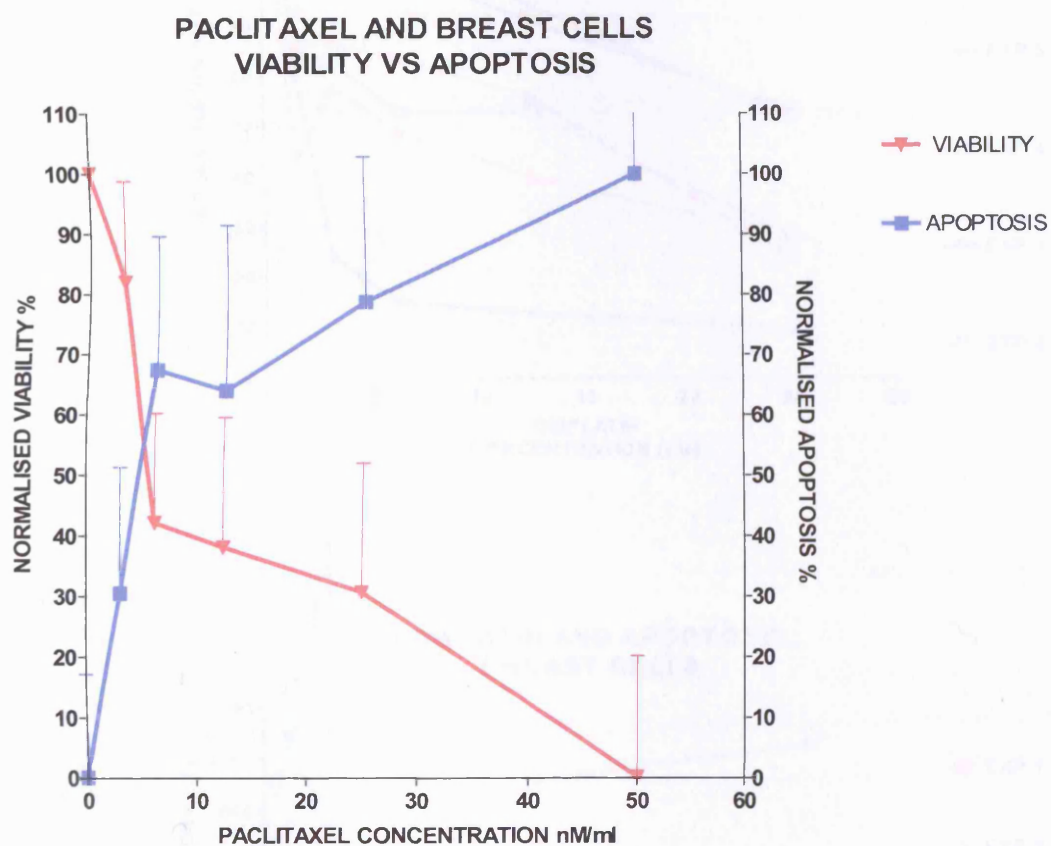


FIGURE 5.2.1.6 Paclitaxel concentration vs. viability and apoptosis in breast cells.

Data points represent the mean and the error bars represent the standard error of the mean (SEM).

CISPLATIN

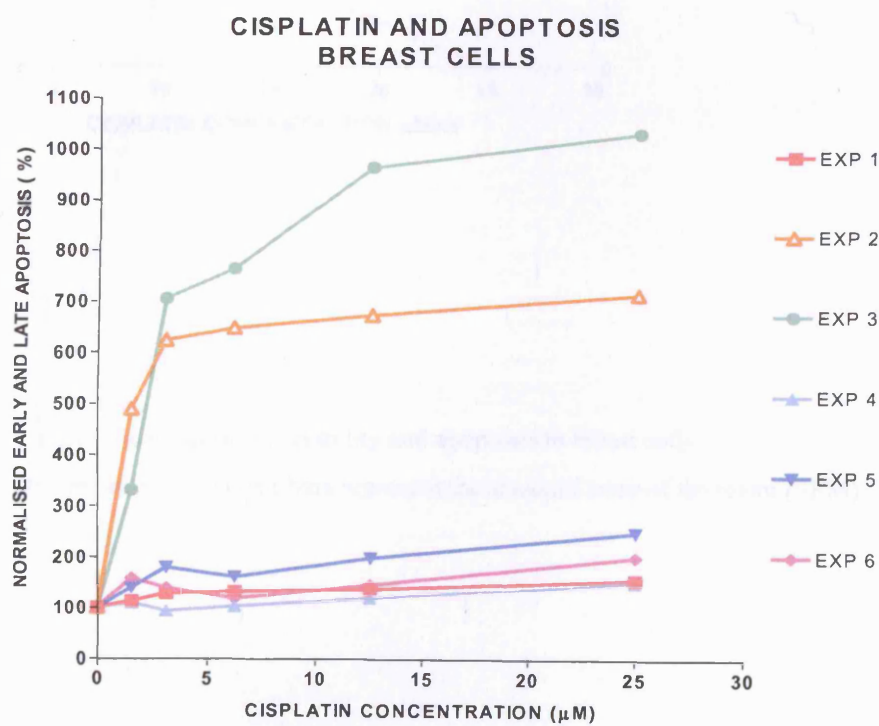
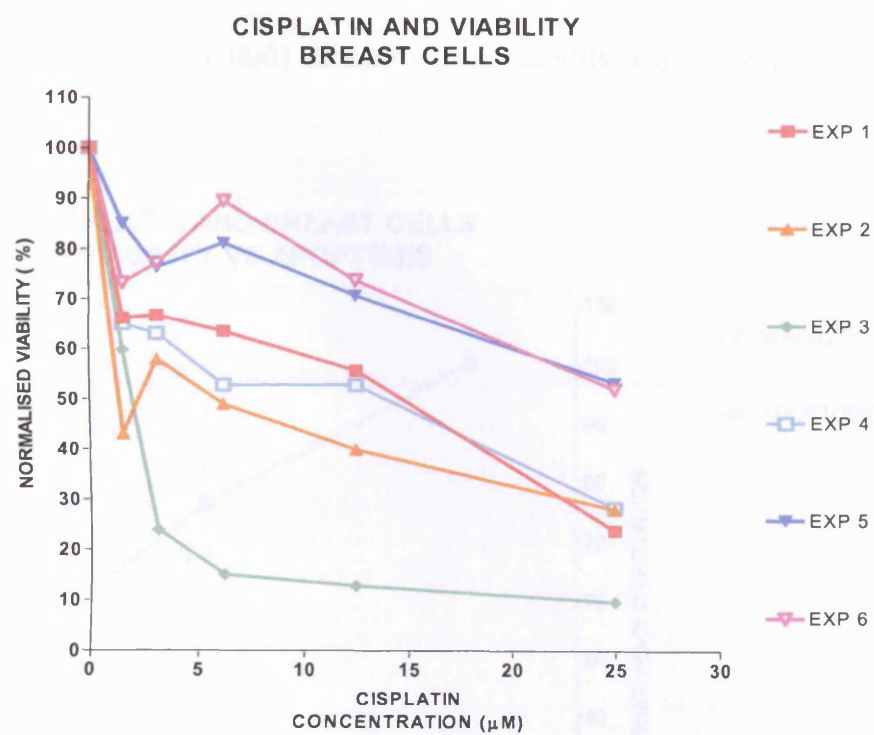


FIGURE 5.2.1.7 Effect of cisplatin on cell viability and apoptosis in MCF-7 cells.

CISPLATIN AND BREAST CELLS - MEANS PLOTS:

Using one-way ANOVA, the overall significance for the difference between groups was $p = 0.002$ for apoptosis and $p = 0.0001$ for normalised viability respectively.

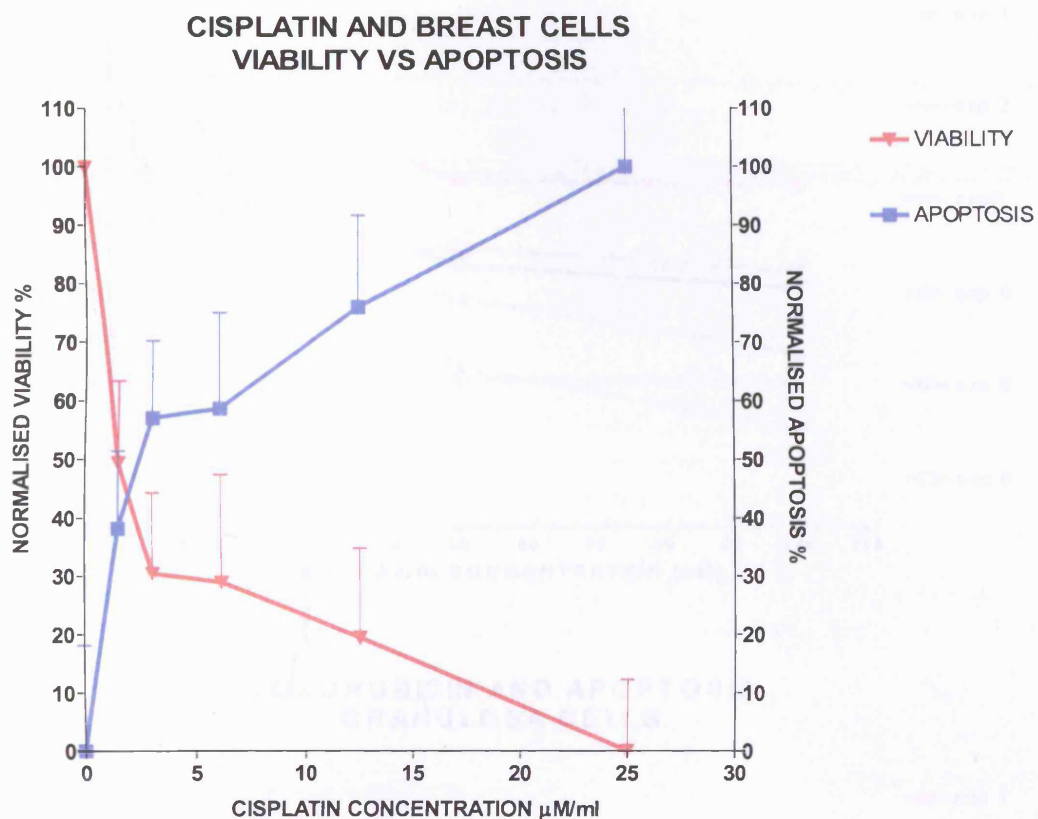


FIGURE 5.2.1.8 Cisplatin concentration vs. viability and apoptosis in breast cells.

Data points represent the mean and the error bars represent the standard error of the mean (SEM).

5.2.2 GRANULOSA CELLS

DOXORUBICIN

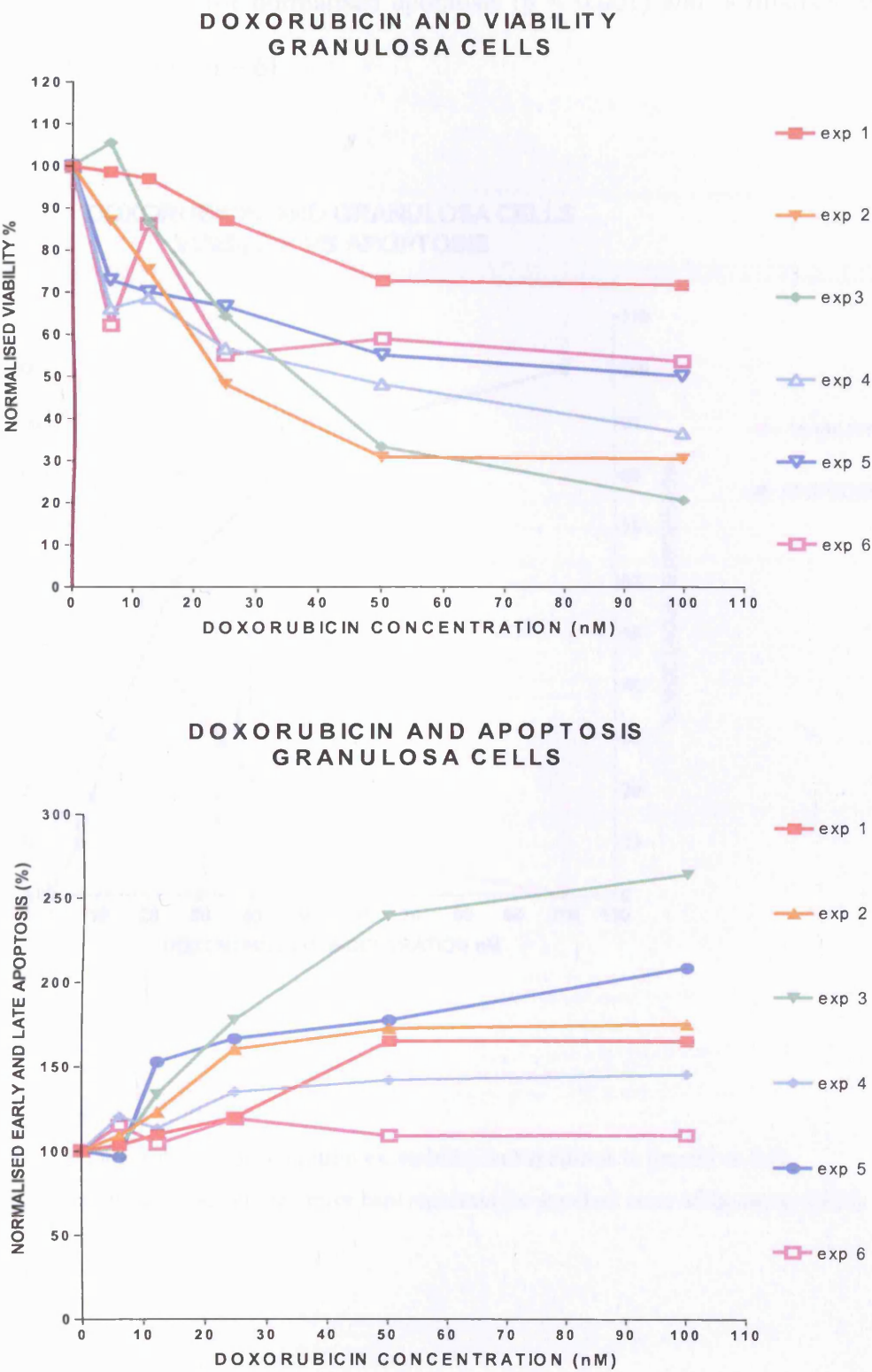


FIGURE 5.2.2.1 Effect of doxorubicin on cell viability and apoptosis in granulosa cells.

DOXORUBICIN AND GRANULOSA CELLS - MEANS PLOTS:

Using one-way ANOVA, there was a significant difference between different concentrations of drug for normalised apoptosis ($p < 0.001$) and normalised viability respectively ($p < 0.001$, $n = 6$).

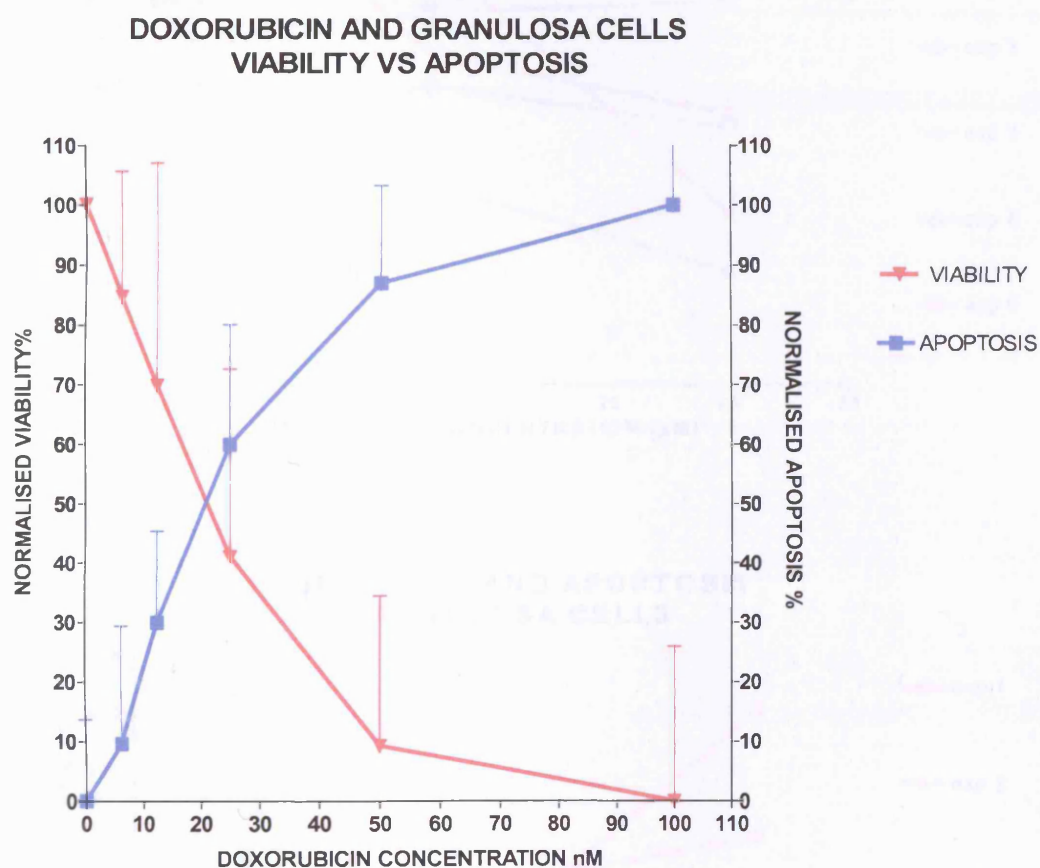


FIGURE 5.2.2.2 Doxorubicin concentration vs. viability and apoptosis in granulosa cells.

Data points represent the mean and the error bars represent the standard error of the mean (SEM).

MELPHALAN

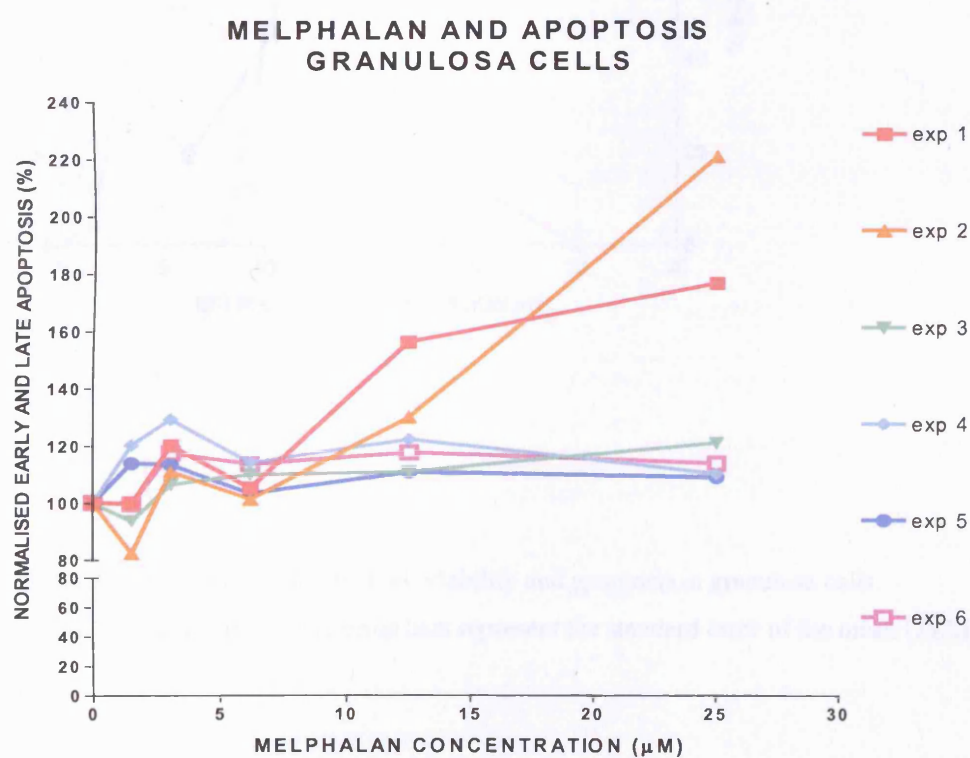
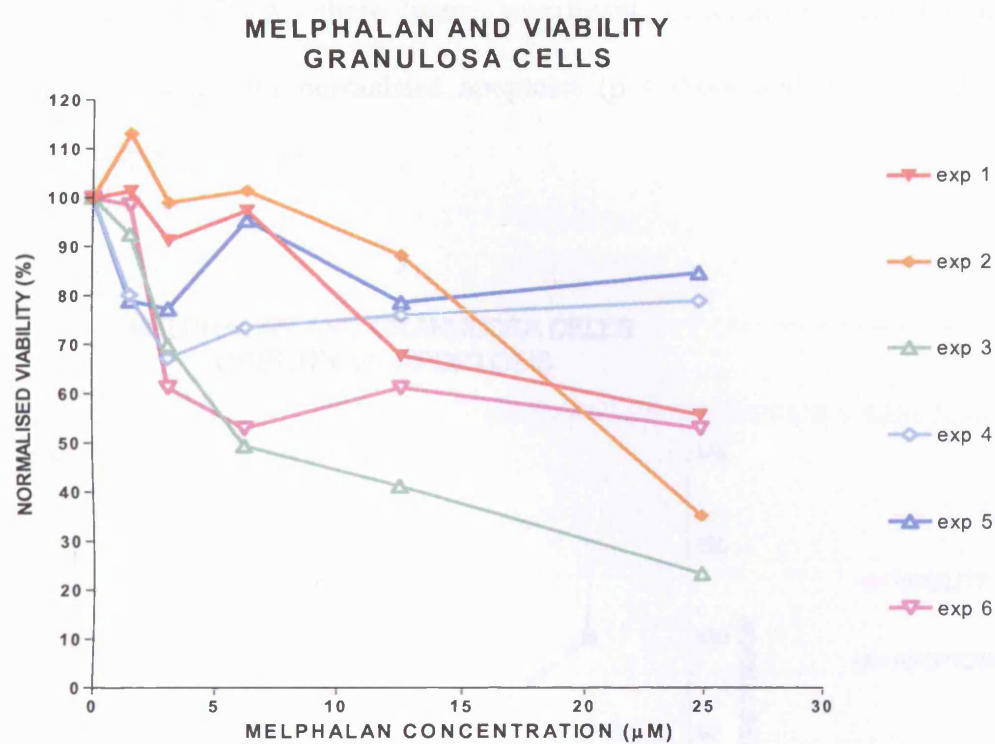


FIGURE 5.2.2.3 Effect of melphalan on cell viability and apoptosis in granulosa cells.

MELPHALAN AND GRANULOSA CELLS - MEANS PLOTS:

Using one-way ANOVA, there were significant differences between different concentrations of drug for normalised apoptosis ($p < 0.05$) and normalised viability respectively ($p < 0.05$, $n = 6$).

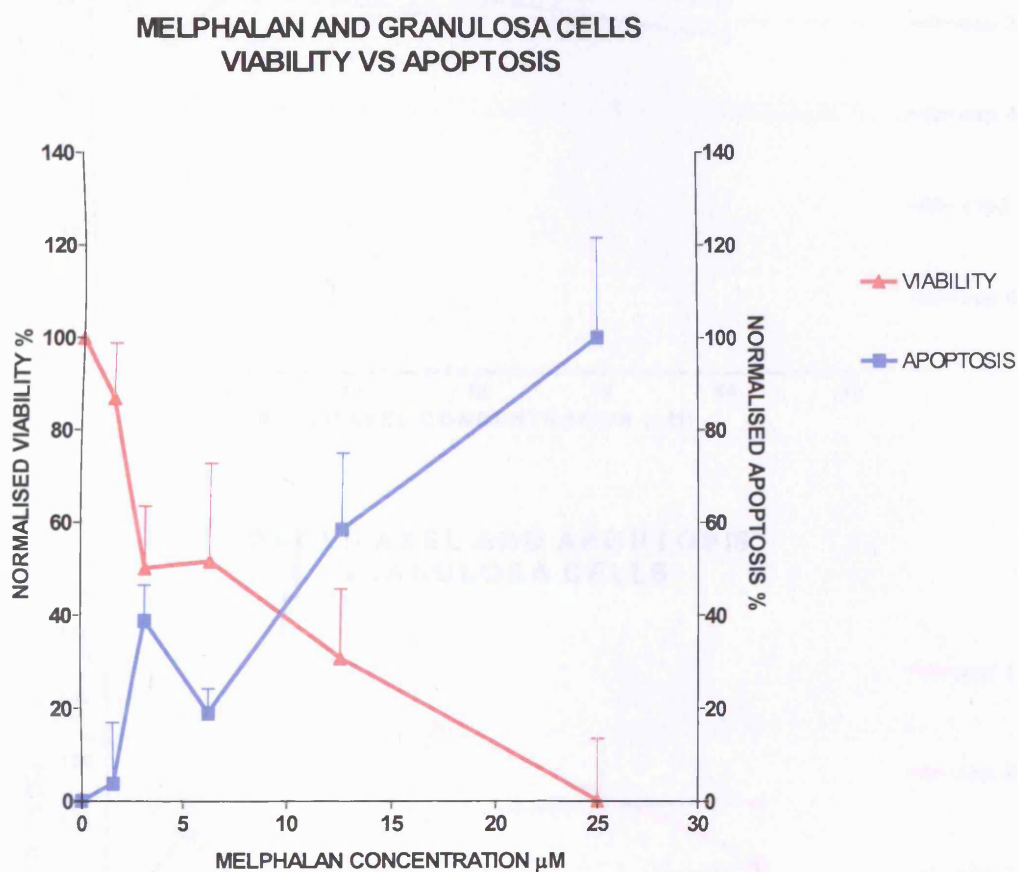


FIGURE 5.2.2.4 Melphalan concentration vs. viability and apoptosis in granulosa cells.

Data points represent the mean and the error bars represent the standard error of the mean (SEM).

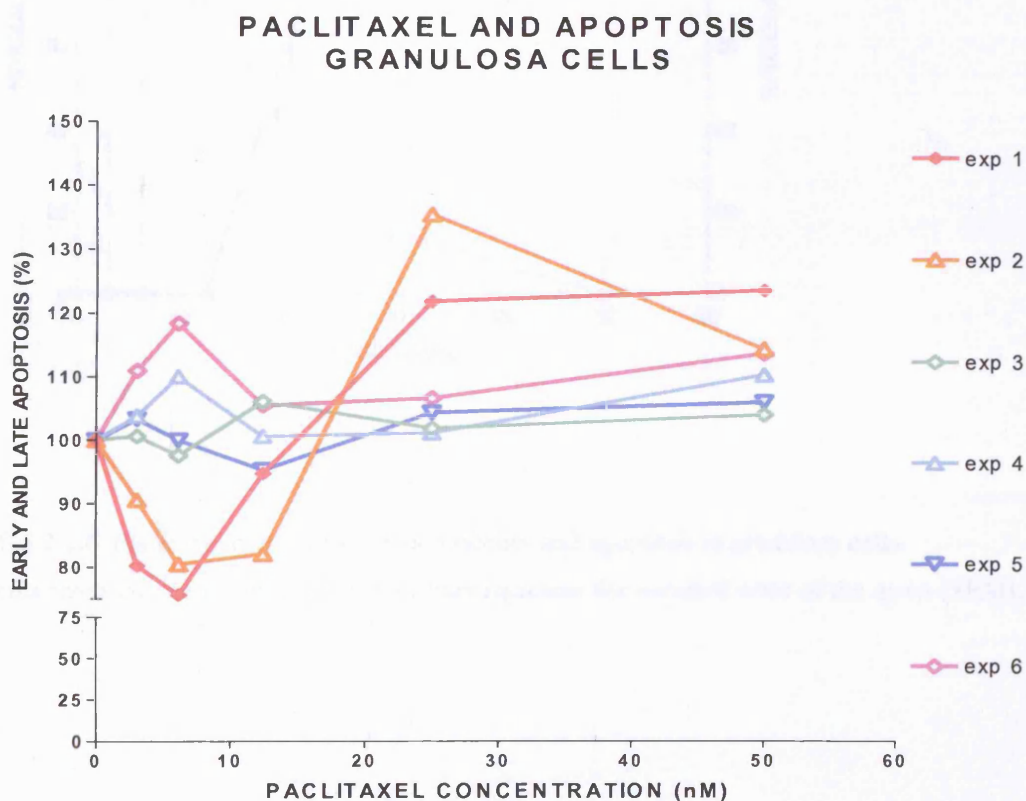
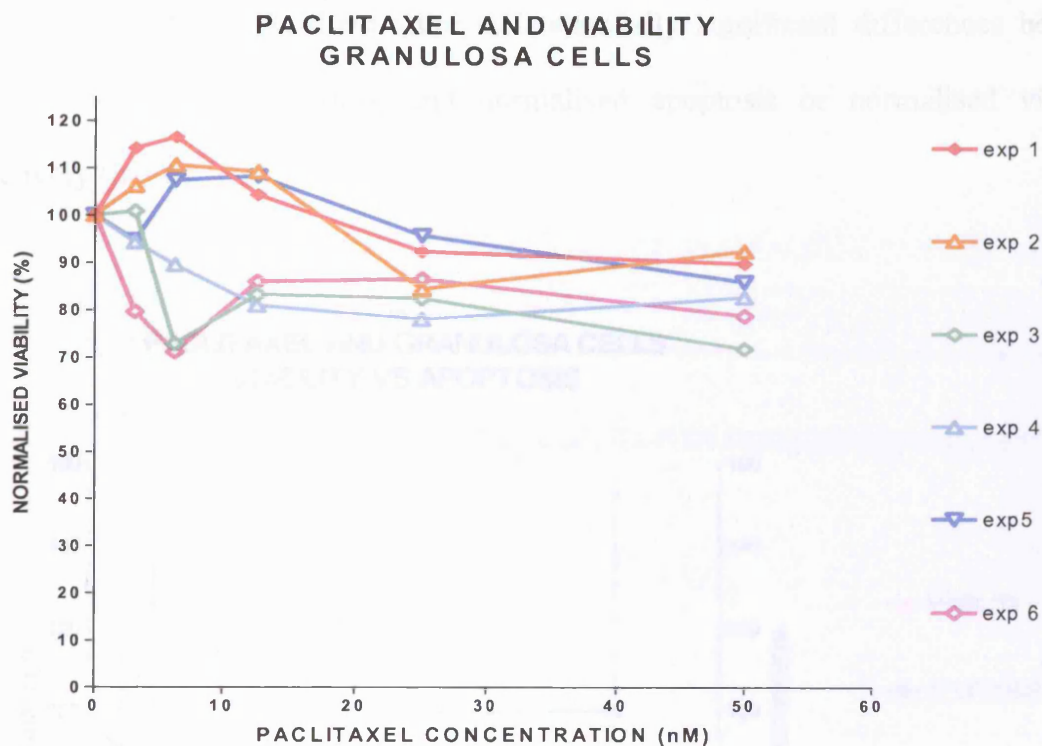


FIGURE 5.2.2.5 Effect of paclitaxel on cell viability and apoptosis in granulosa cells.

PACLITAXEL AND GRANULOSA CELLS - MEANS PLOTS:

Using one-way ANOVA, there were no statistically significant differences between various concentrations of drug and normalised apoptosis or normalised viability respectively ($n = 6$).

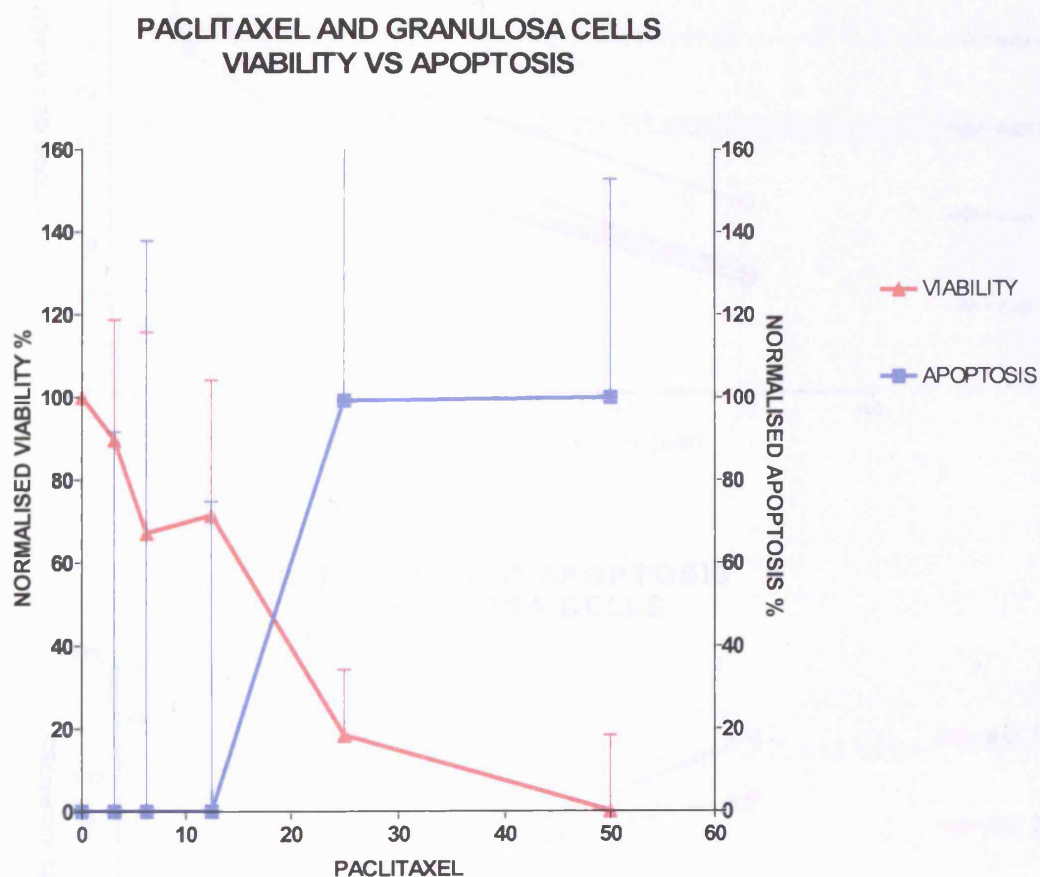


FIGURE 5.2.2.6 Paclitaxel concentration vs. viability and apoptosis in granulosa cells.

Data points represent the mean and the error bars represent the standard error of the mean (SEM).

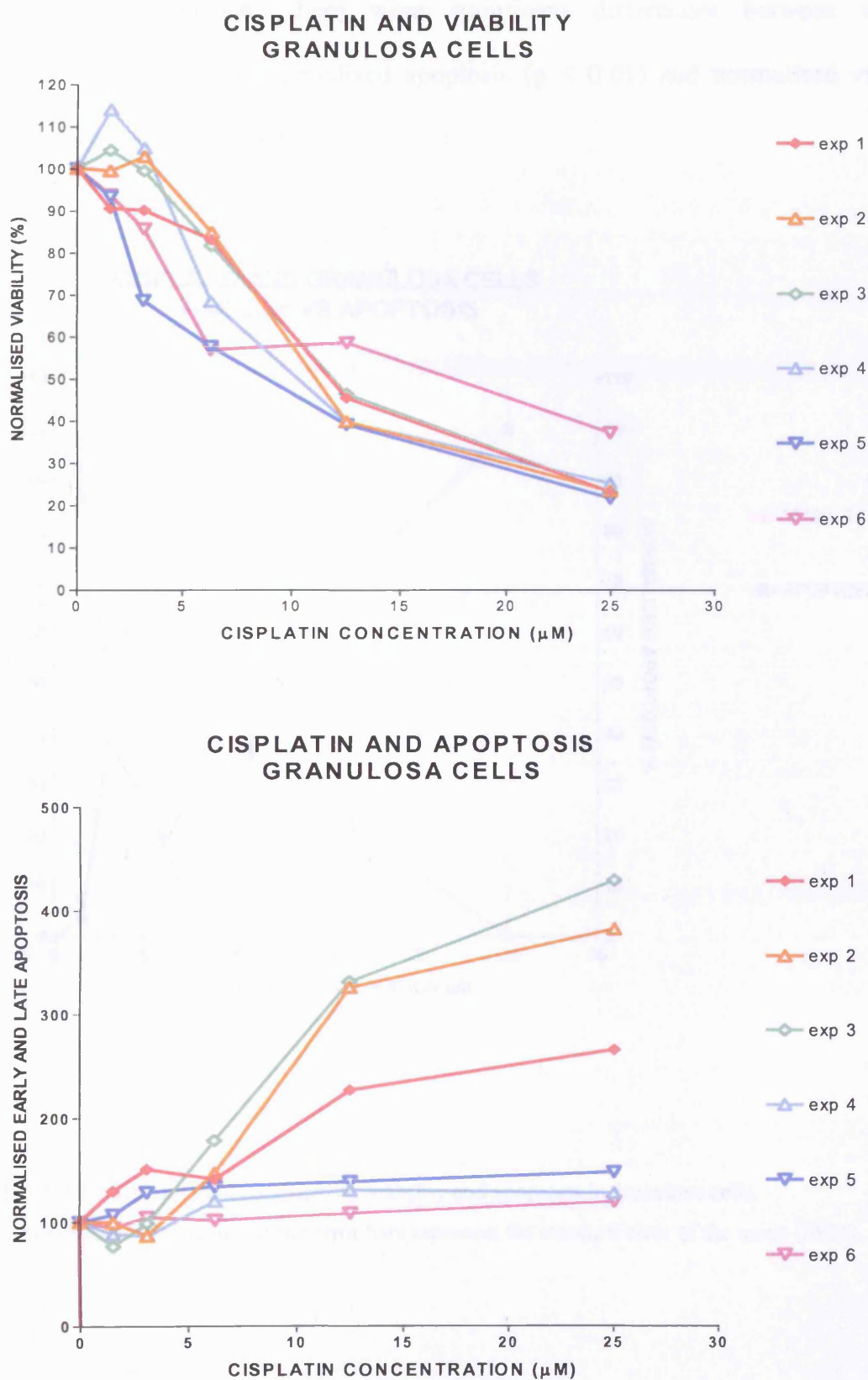


FIGURE 5.2.2.7 Effect of cisplatin on cell viability and apoptosis in granulosa cells.

CISPLATIN AND GRANULOSA CELLS - MEANS PLOTS:

Using one-way ANOVA, there were significant differences between various concentrations of drug for normalised apoptosis ($p < 0.01$) and normalised viability respectively ($p < 0.001$, $n = 6$).

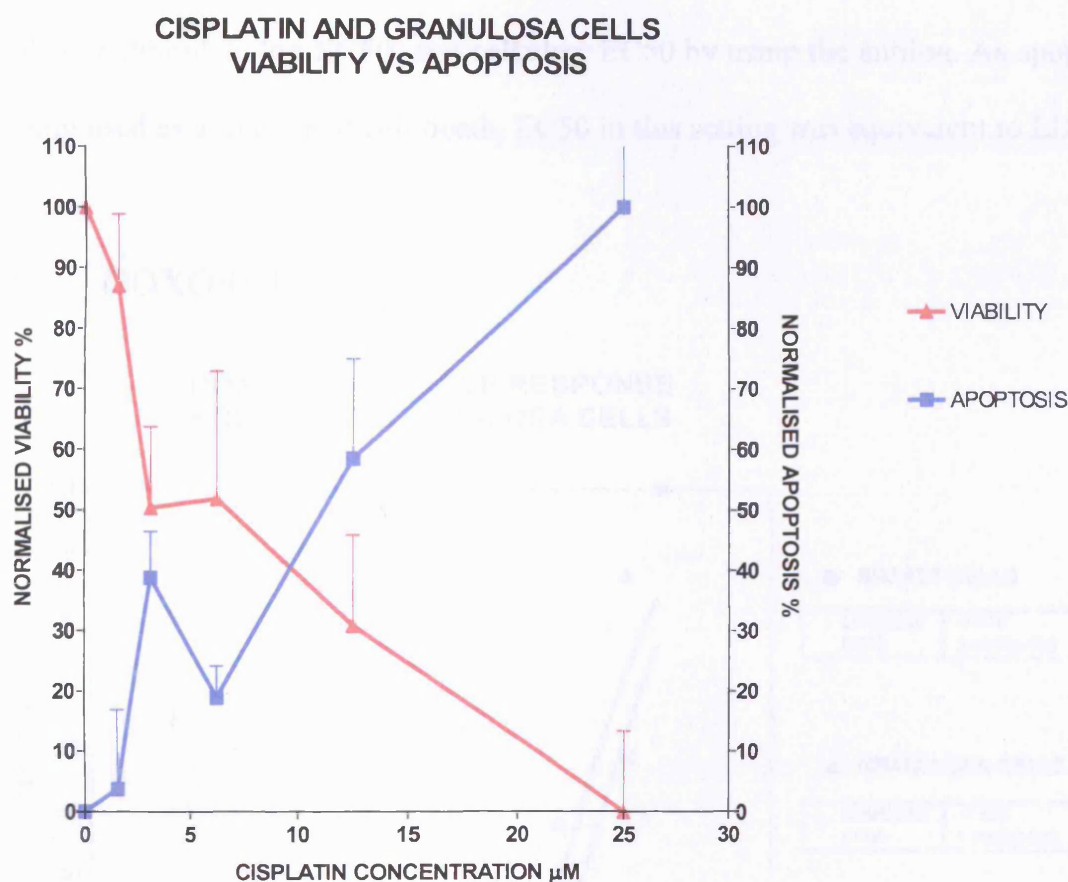


FIGURE 5.2.2.8 Cisplatin concentration vs. viability and apoptosis in granulosa cells.

Data points represent the mean and the error bars represent the standard error of the mean (SEM).

5.3 DOSE RESPONSE

5.3.1 INTRODUCTION

Based on the means plots obtained from drug concentration vs. normalised viability and apoptosis respectively, non-linear regression was used to establish sigmoid dose-response curves for breast and granulosa cells respectively. From these curves, it was possible to extrapolate log EC50, and calculate EC50 by using the antilog. As apoptosis was being used as a marker of cell death, EC50 in this setting was equivalent to LD50.

5.3.2 DOXORUBICIN

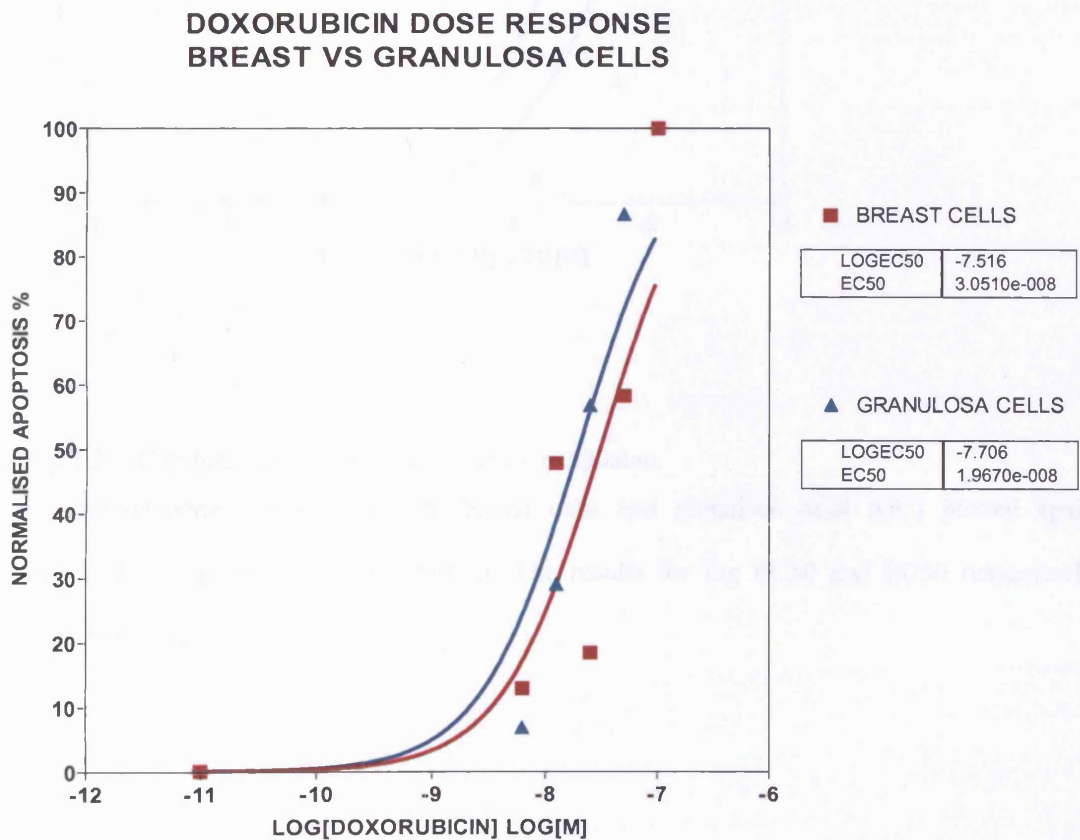


FIGURE 5.3.1 Combined dose response curves – doxorubicin. Sigmoidal dose-response curves for both breast cells and granulosa cells (GC) plotted against a logarithmic scale for doxorubicin concentration. The results for log EC50 and EC50 respectively are displayed on the graph.

5.3.3 MELPHALAN

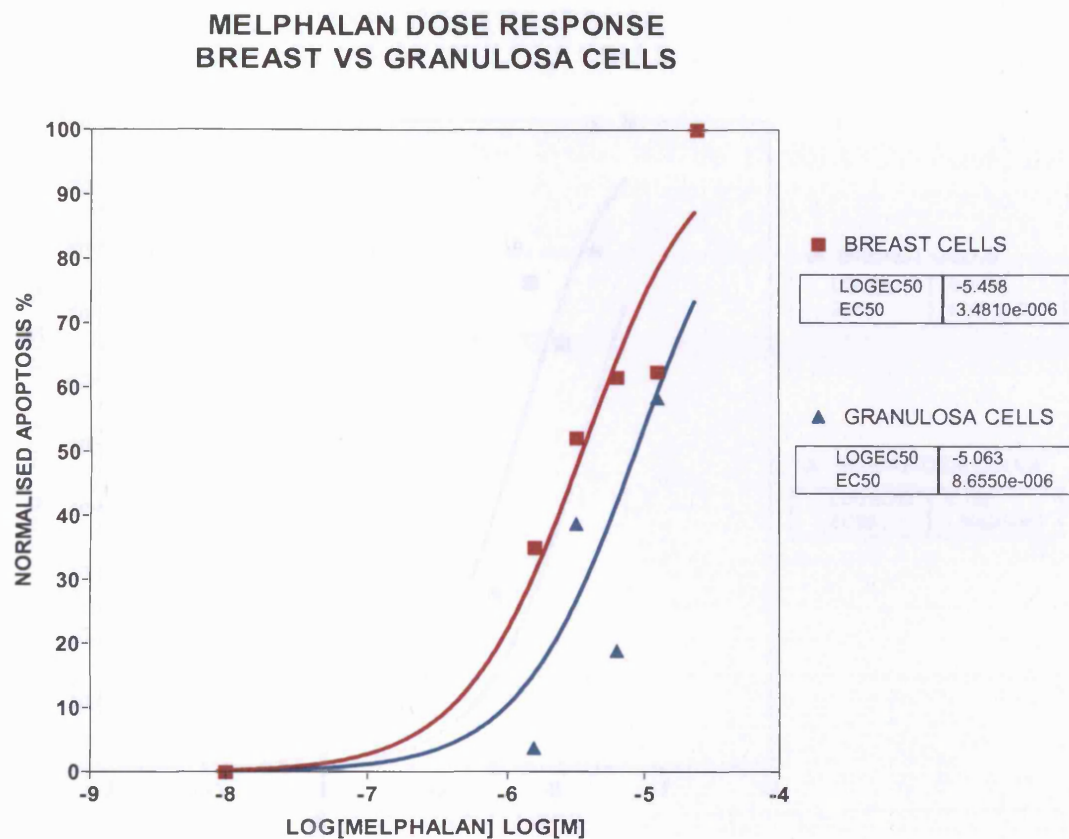


FIGURE 5.3.2 Combined dose response curves – melphalan.

Sigmoidal dose-response curves for both breast cells and granulosa cells (GC) plotted against a logarithmic scale for melphalan concentration. The results for log EC50 and EC50 respectively are displayed on the graph.

5.3.4 PACLITAXEL

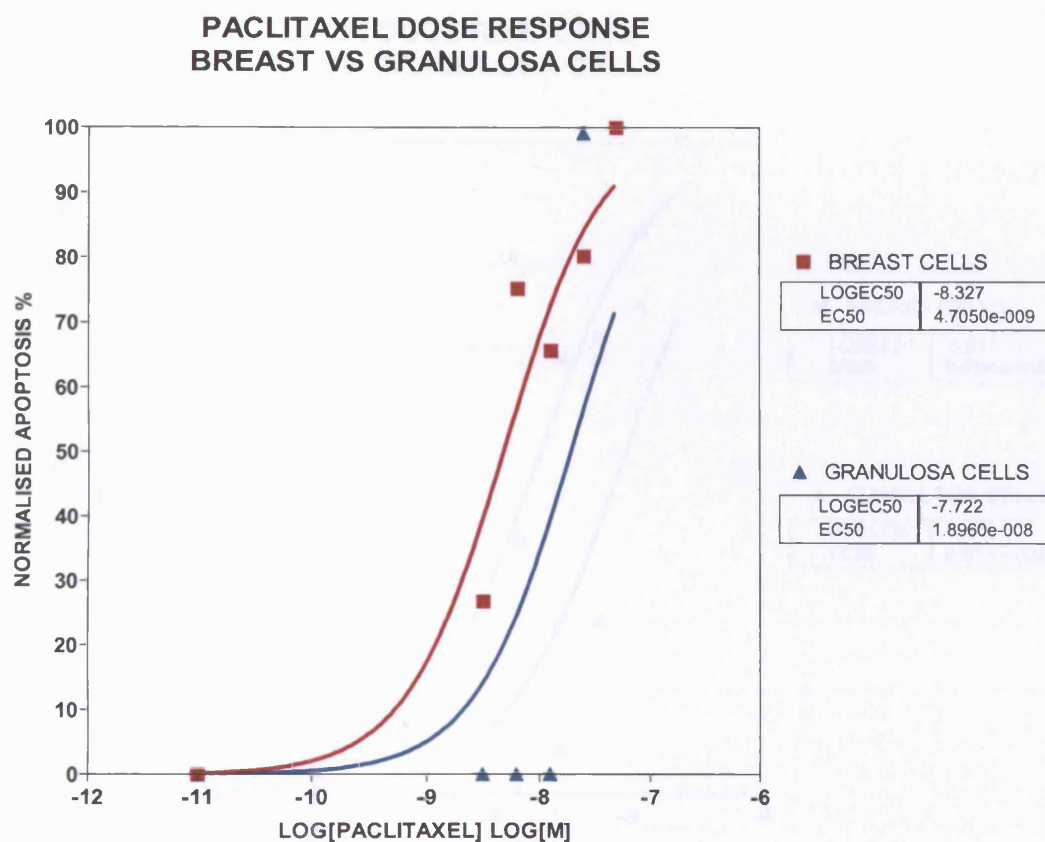


FIGURE 5.3.3 Combined dose response curves – paclitaxel.

Sigmoidal dose-response curves for both breast cells and granulosa cells (GC) plotted against a logarithmic scale for Paclitaxel concentration. The results for log EC50 and EC50 respectively are displayed on the graph.

5.3.5 CISPLATIN

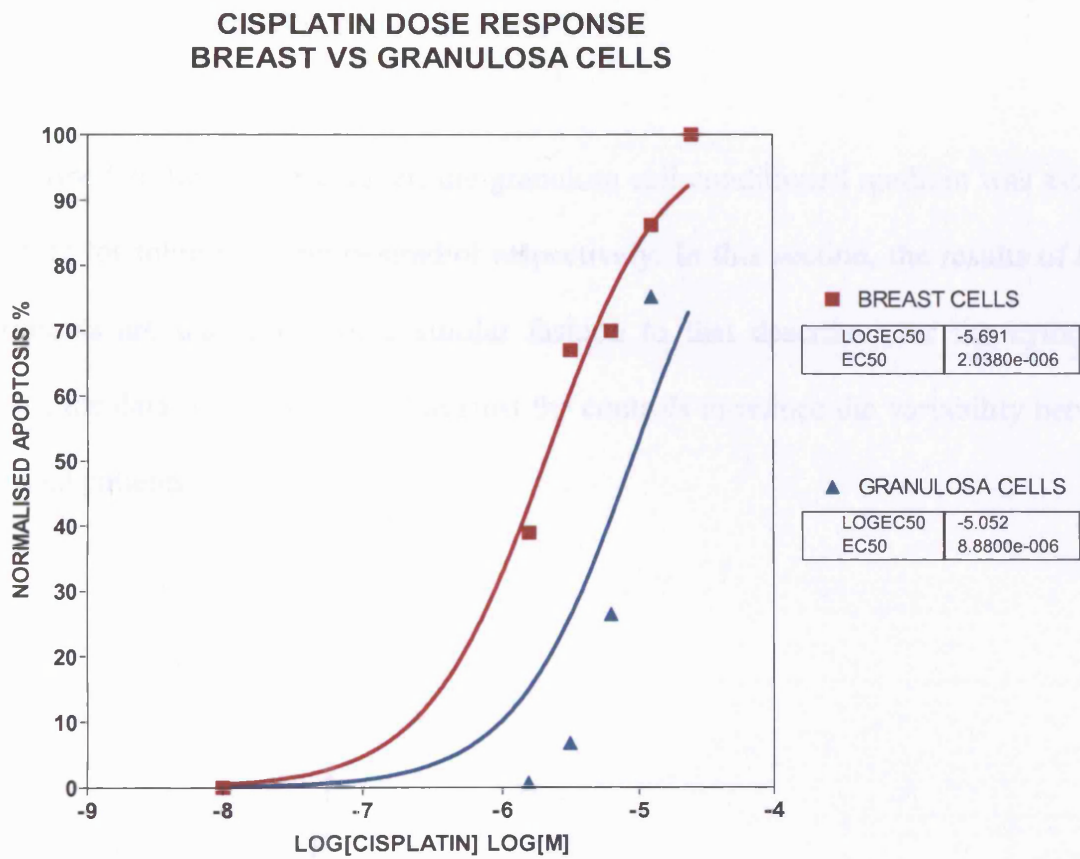


FIGURE 5.3.4 Combined dose response curves – cisplatin. Sigmoidal dose-response curves for both breast cells and granulosa cells (GC) plotted against a logarithmic scale for Cisplatin concentration. The results for log EC50 and EC50 respectively are displayed on the graph.

5.4 HORMONE RESULTS

5.4.1 GRANULOSA CELLS

5.4.1.1 INTRODUCTION

As described earlier in the chapter, the granulosa cell conditioned medium was assayed separately for inhibin A and oestradiol respectively. In this section, the results of these experiments are displayed. In a similar fashion to that described for the cytometry analysis, the data were normalised against the controls to reduce the variability between individual patients.

5.4.1.2 DOXORUBICIN

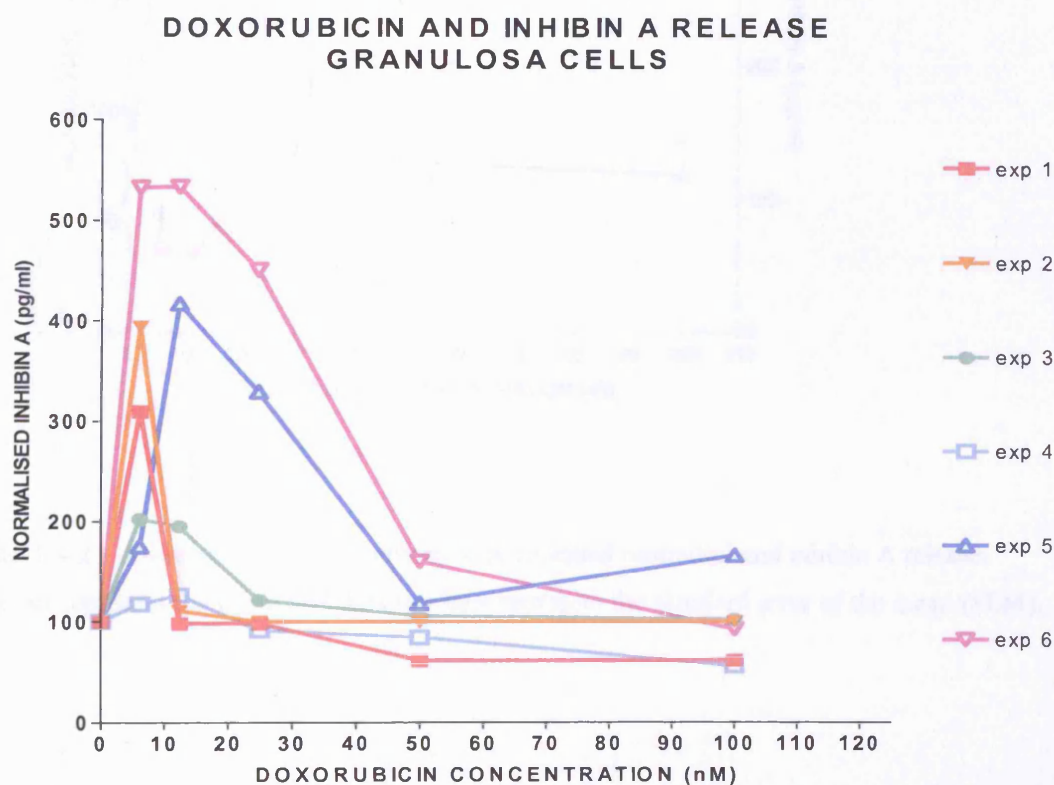
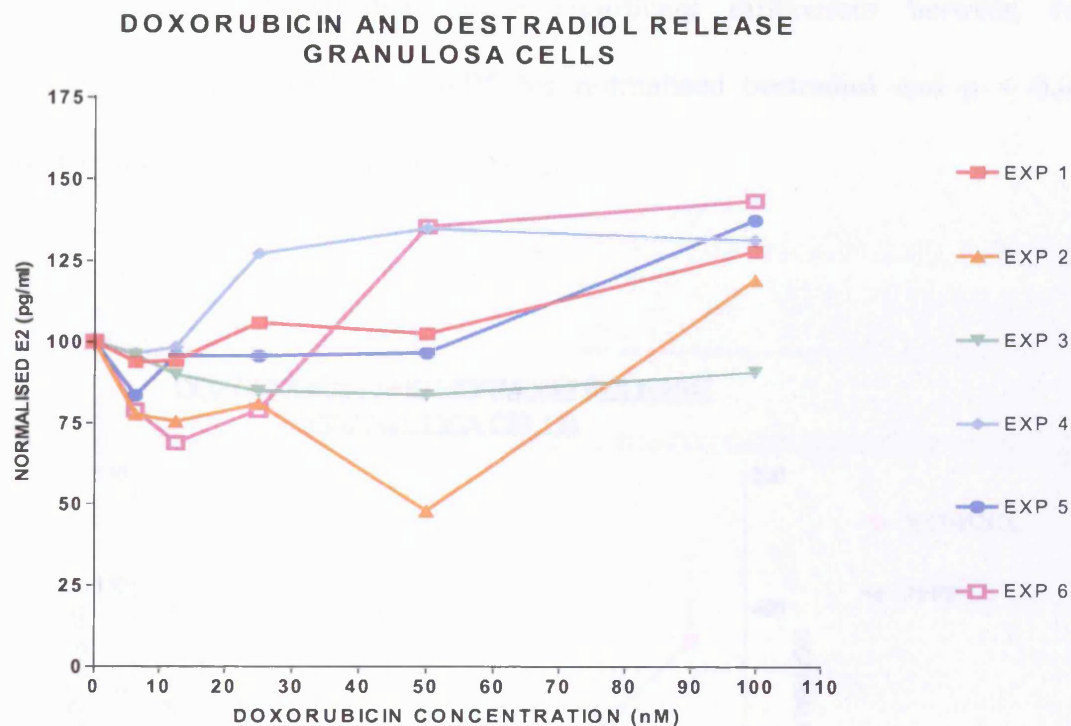


FIGURE 5.4.1 Granulosa cell hormone release – doxorubicin.

Normalised oestradiol and inhibin A quantified from the media used to culture the granulosa cells at different concentrations of doxorubicin.

DOXORUBICIN AND HORMONE RELEASE – MEANS PLOTS:

Using one-way ANOVA, there were significant differences between various concentrations of drug with $p < 0.05$ for normalised oestradiol and $p < 0.01$ for normalised inhibin A respectively ($n = 6$).

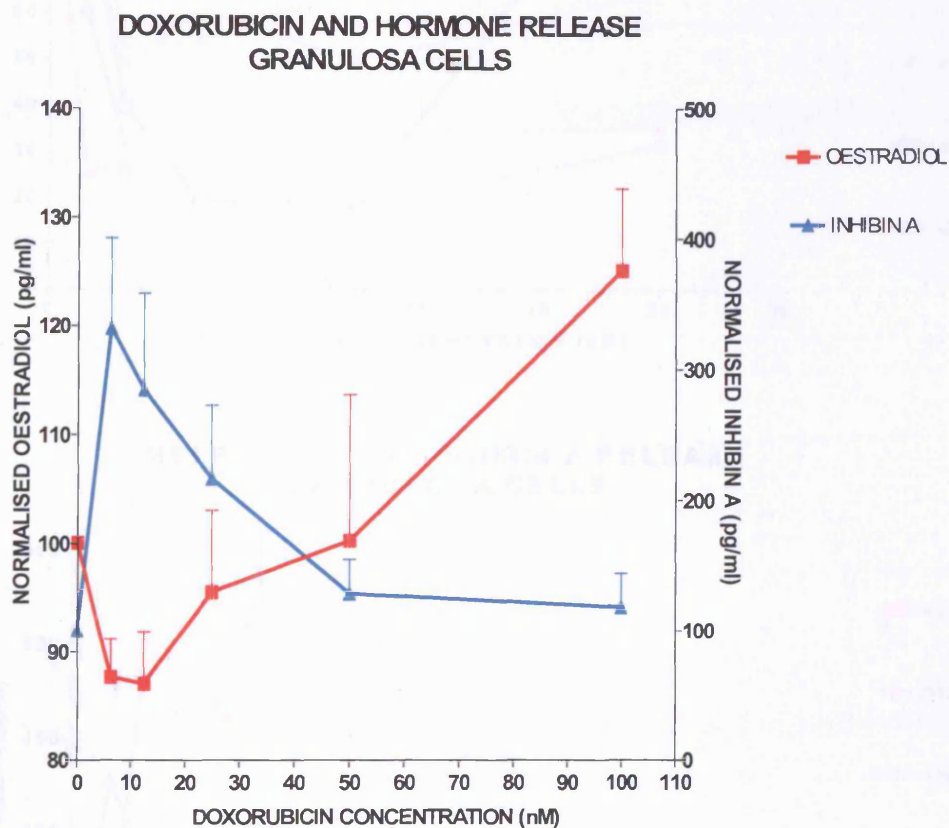


FIGURE 5.4.2 Doxorubicin concentration vs. normalised oestradiol and inhibin A release.

Data points represent the mean and the error bars represent the standard error of the mean (SEM).

5.4.1.3 MELPHALAN

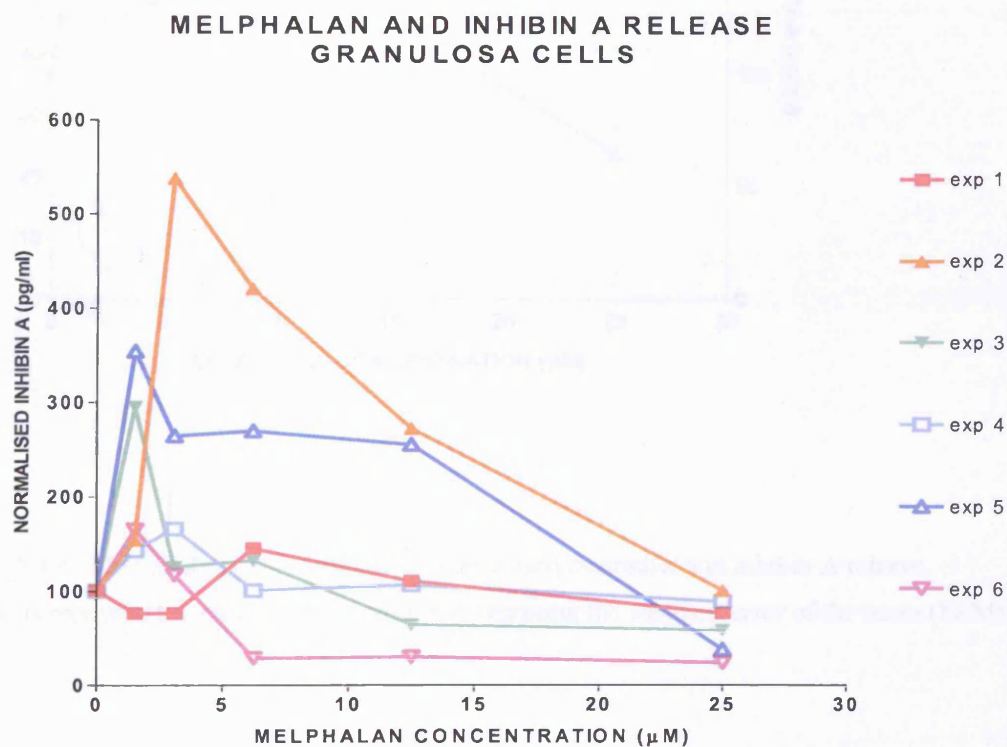
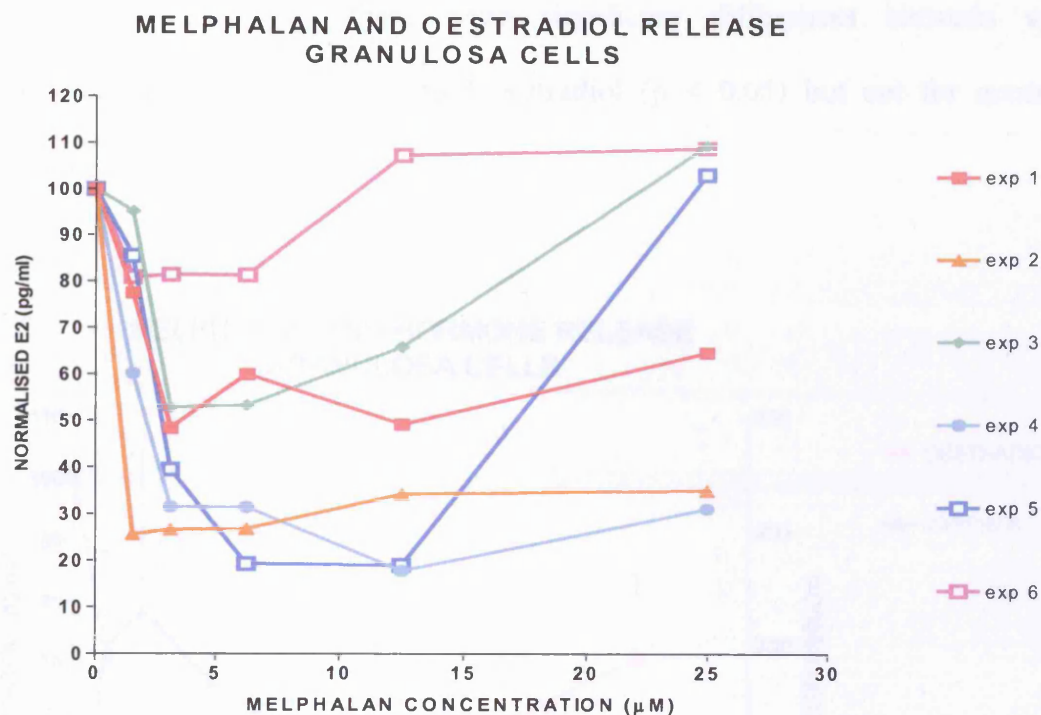


FIGURE 5.4.3 Granulosa cell hormone release – melphalan.

Normalised oestradiol and inhibin A quantified from the media used to culture the granulosa cells at different concentrations of melphalan.

MELPHALAN AND HORMONE RELEASE – MEANS PLOTS:

Using one-way ANOVA, there were significant differences between various concentrations of drug for normalised oestradiol ($p < 0.05$) but not for normalised inhibin A ($n = 6$).

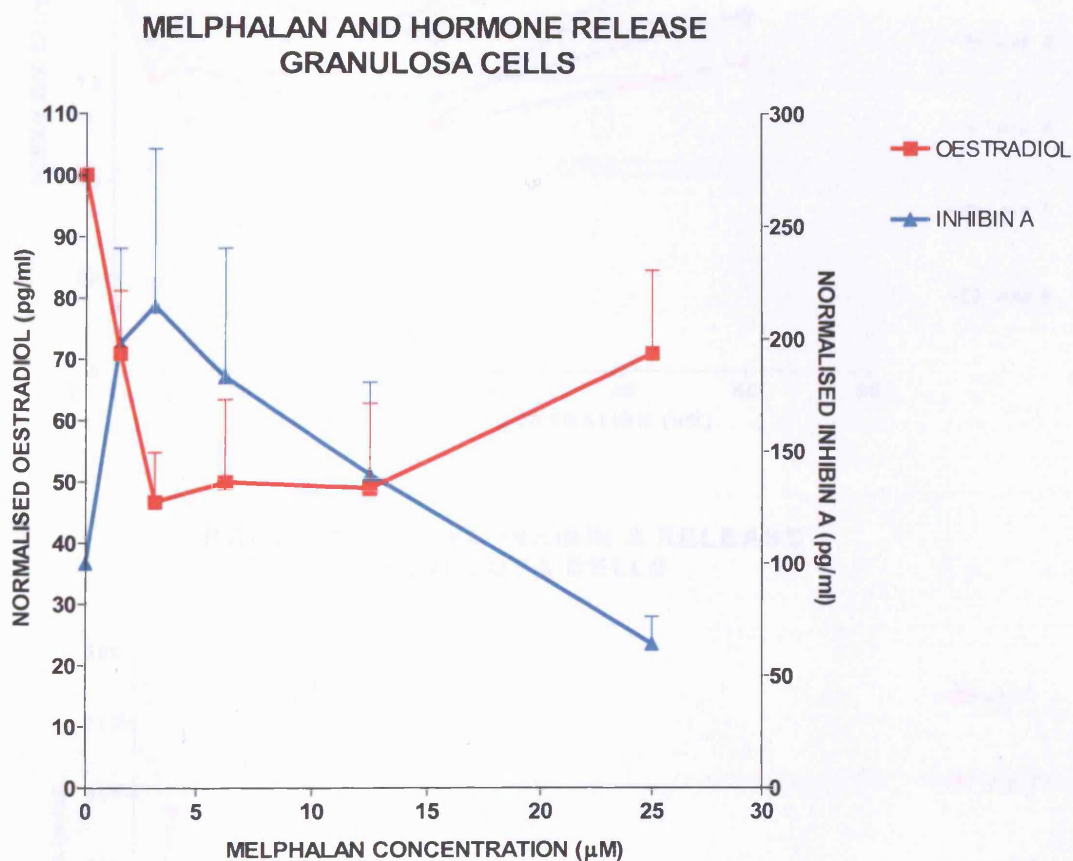


FIGURE 5.4.4 Melphalan concentration vs. normalised oestradiol and inhibin A release.

Data points represent the mean and the error bars represent the standard error of the mean (SEM).

5.4.1.4 PACLITAXEL

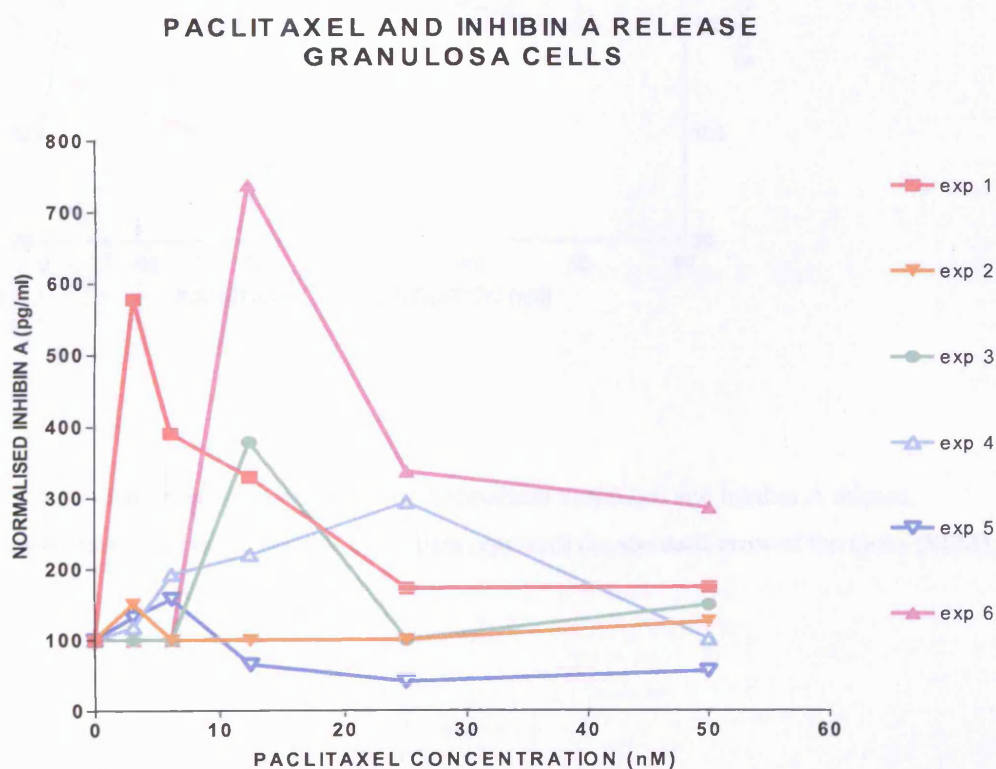
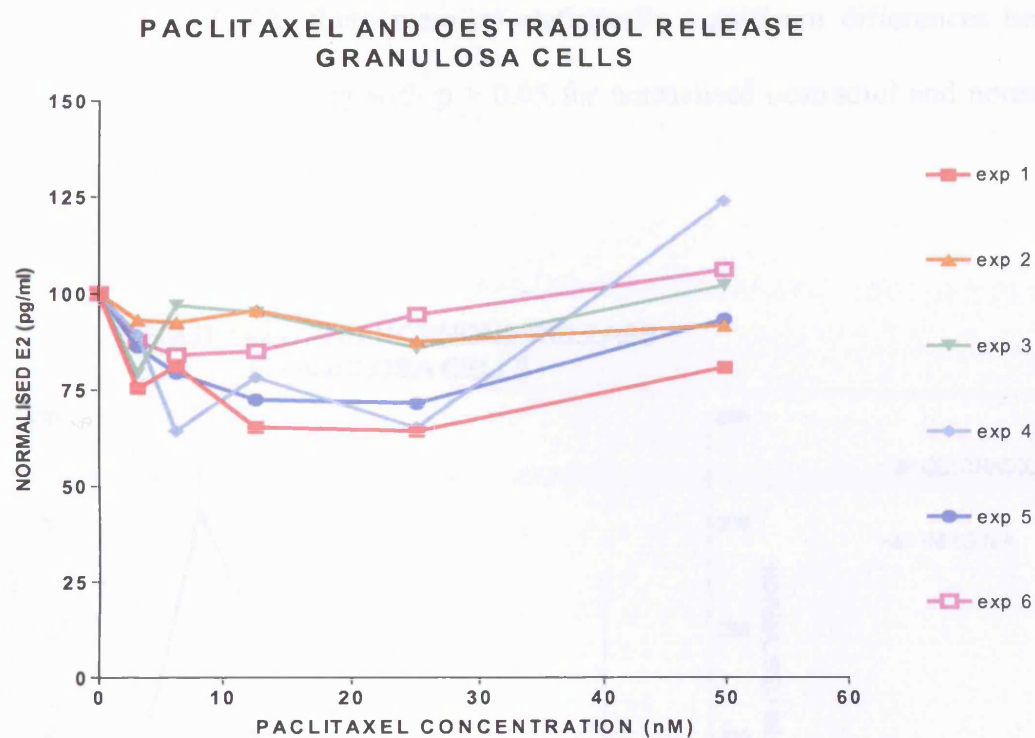


FIGURE 5.4.5 Granulosa cell hormone release – paclitaxel.

Normalised oestradiol and inhibin A quantified from the media used to culture the granulosa cells at different concentrations of paclitaxel.

PACLITAXEL AND HORMONE RELEASE – MEANS PLOTS:

Using one-way ANOVA, there were no statistically significant differences between various concentrations of drug with $p > 0.05$ for normalised oestradiol and normalised inhibin A respectively ($n = 6$).

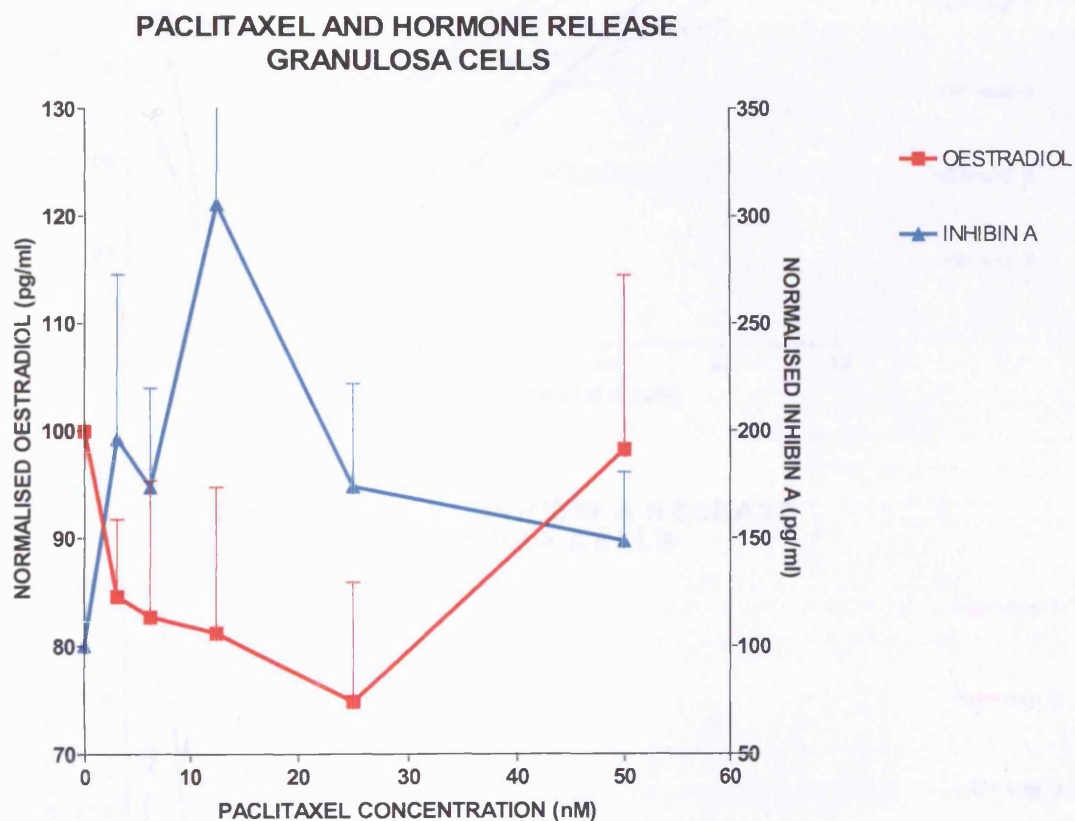


FIGURE 5.4.6 Paclitaxel concentration vs. normalised oestradiol and inhibin A release.

Data points represent the mean and the error bars represent the standard error of the mean (SEM).

5.4.1.5 CISPLATIN

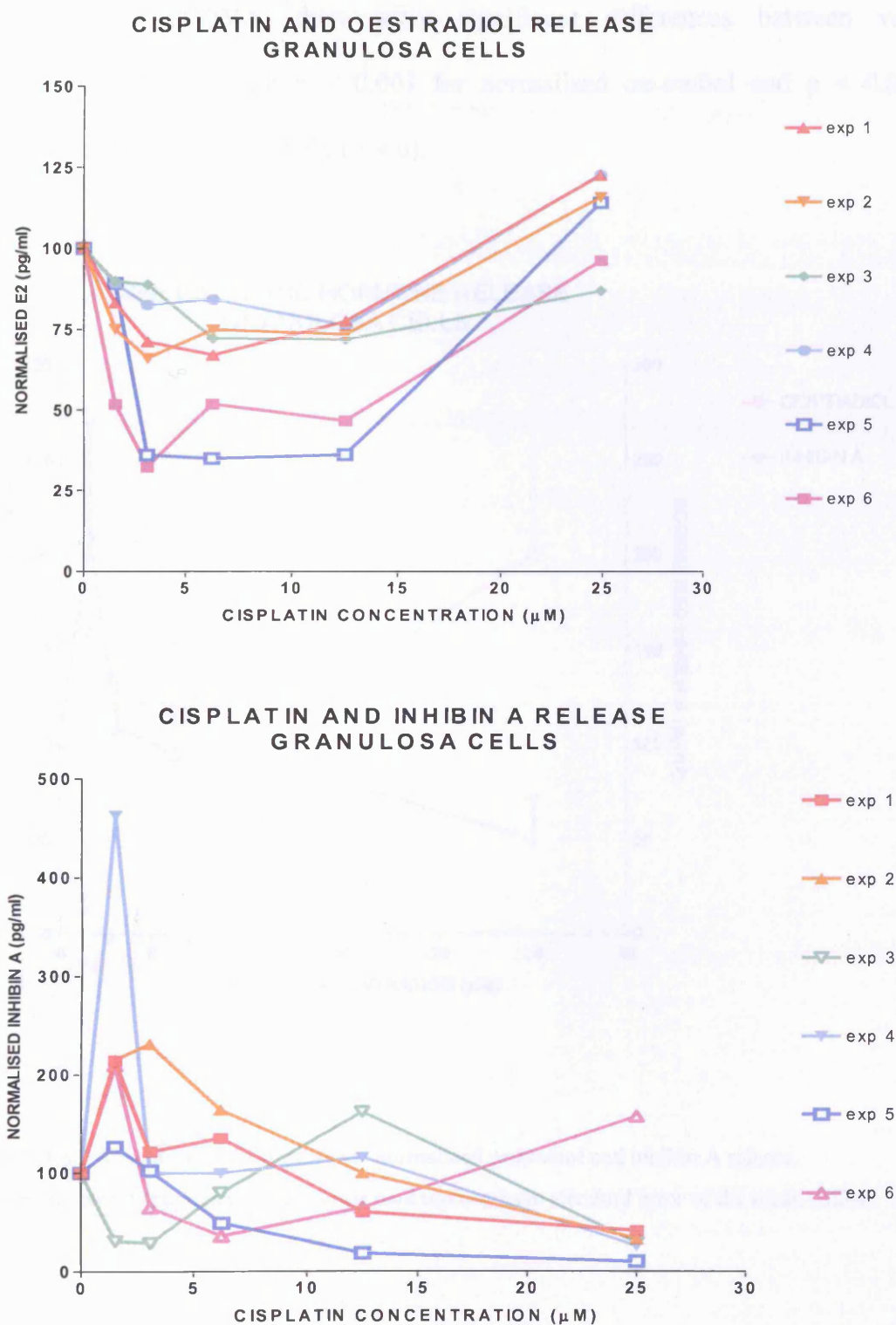


FIGURE 5.4.7 Granulosa cell hormone release – cisplatin.

Normalised oestradiol and inhibin A quantified from the media used to culture the granulosa cells at different concentrations of cisplatin.

CISPLATIN AND HORMONE RELEASE – MEANS PLOTS:

Using one-way ANOVA, there were significant differences between various concentrations of drug with $p < 0.001$ for normalised oestradiol and $p < 0.05$ for normalised inhibin A respectively ($n = 6$).

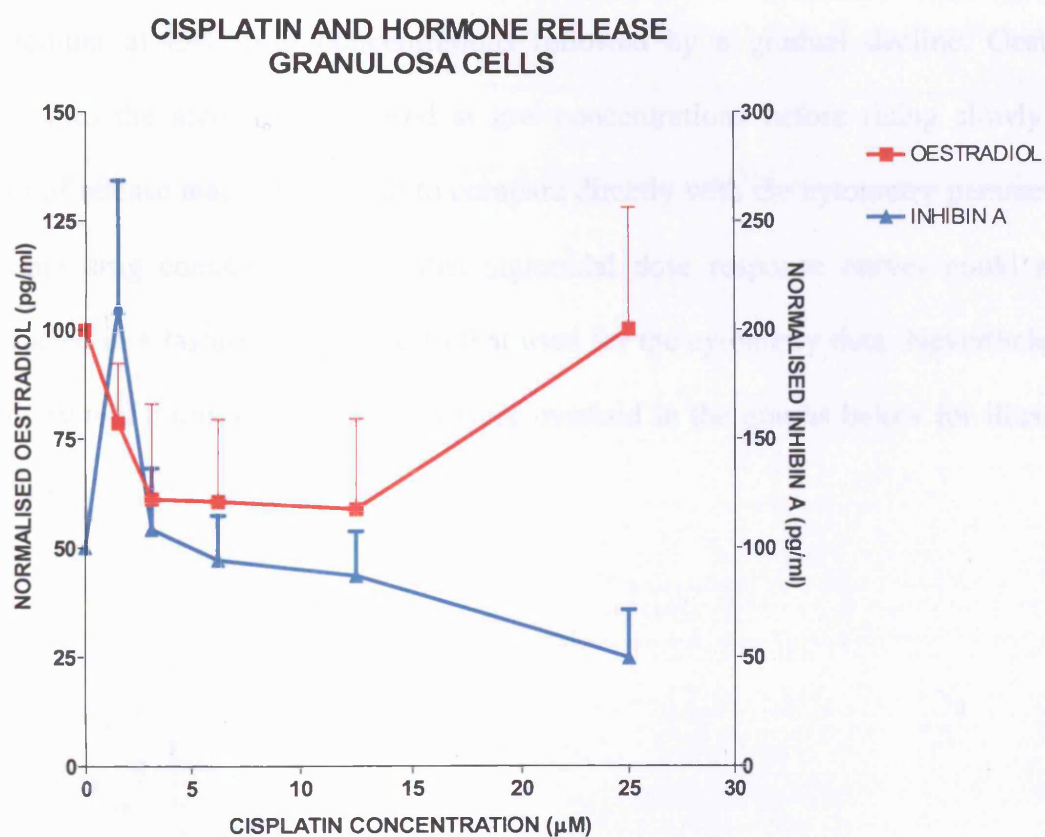


FIGURE 5.4.8 Cisplatin concentration vs. normalised oestradiol and inhibin A release.

Data points represent the mean and the error bars represent the standard error of the mean (SEM).

5.5 COMBINED DATA: CYTOMETRY AND HORMONES

5.5.1 INTRODUCTION

The combined cytometry and hormone release data after drug administration for the granulosa cells will be displayed. Hormone release from the granulosa cells appeared to be biphasic. In the case of inhibin A release, there appeared to be an initial surge into the medium at low drug concentrations followed by a gradual decline. Oestradiol release into the medium decreased at low concentrations before rising slowly. This pattern of release made it difficult to compare directly with the cytometry parameters at the same drug concentration, in that sigmoidal dose response curves could not be constructed in a fashion analogous to that used for the cytometry data. Nevertheless the cytometry and hormone release data were overlaid in the graphs below for illustrative purposes.

5.5.2 DOXORUBICIN

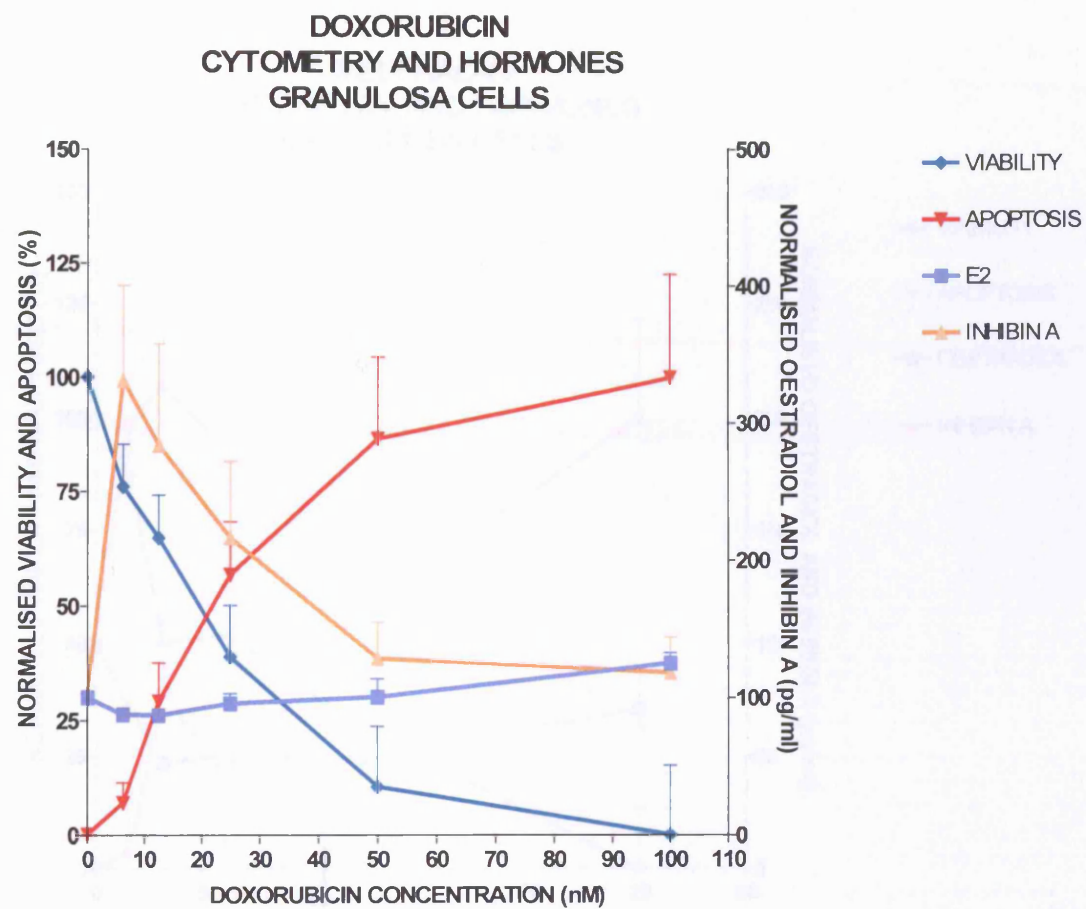


FIGURE 5.5.1 Combined cytometry and hormone data for granulosa cells – doxorubicin.
Cytometry and hormone data overlaid using a doubly Y-axis against doxorubicin concentration.

5.5.3 MELPHALAN

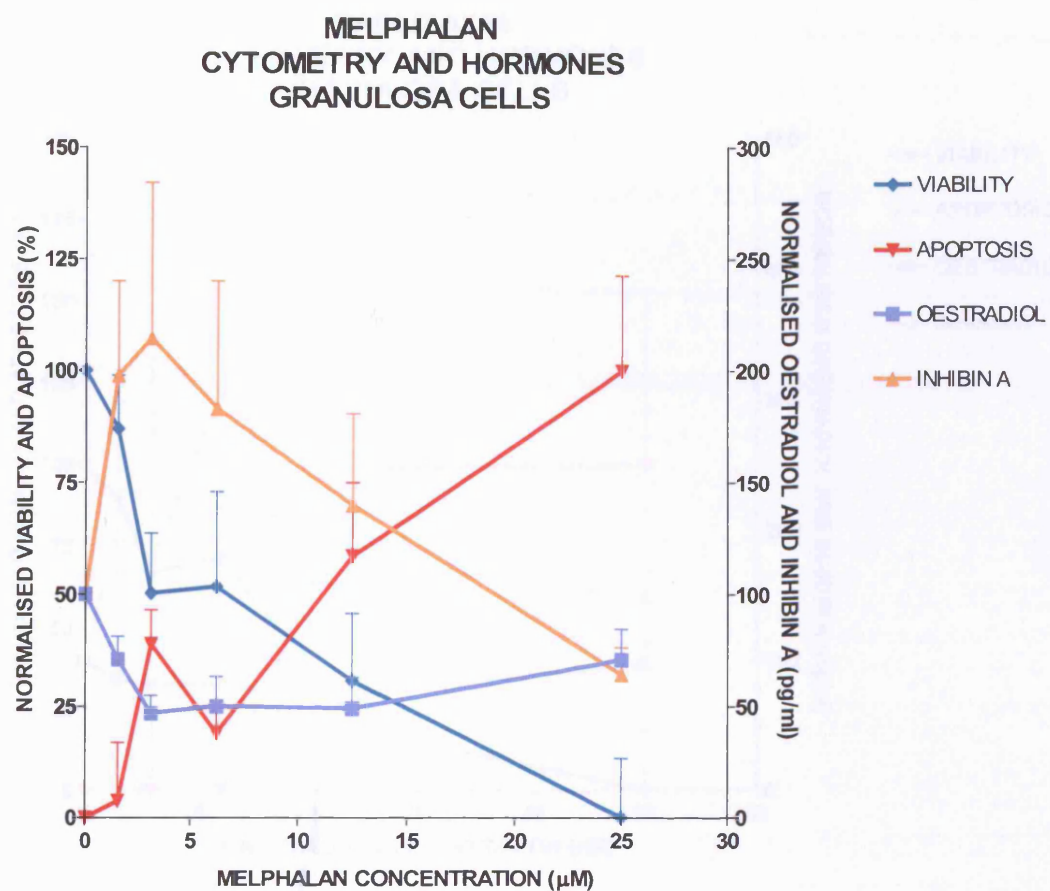


FIGURE 5.5.2 Combined cytometry and hormone data for granulosa cells – melphalan.
Cytometry and hormone data overlaid using a doubly Y-axis against melphalan concentration.

5.5.4 PACLITAXEL

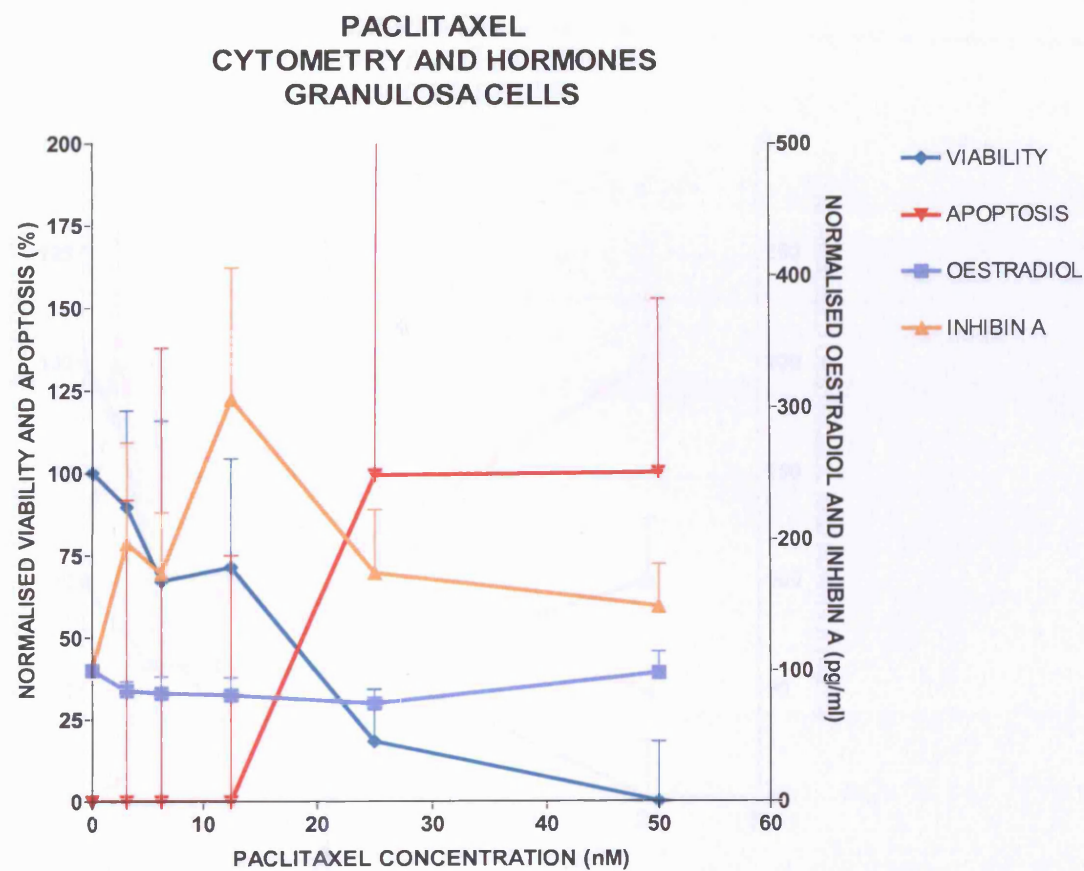


FIGURE 5.5.3 Combined cytometry and hormone data for granulosa cells – paclitaxel.
Cytometry and hormone data overlaid using a doubly Y-axis against paclitaxel concentration.

5.5.5 CISPLATIN

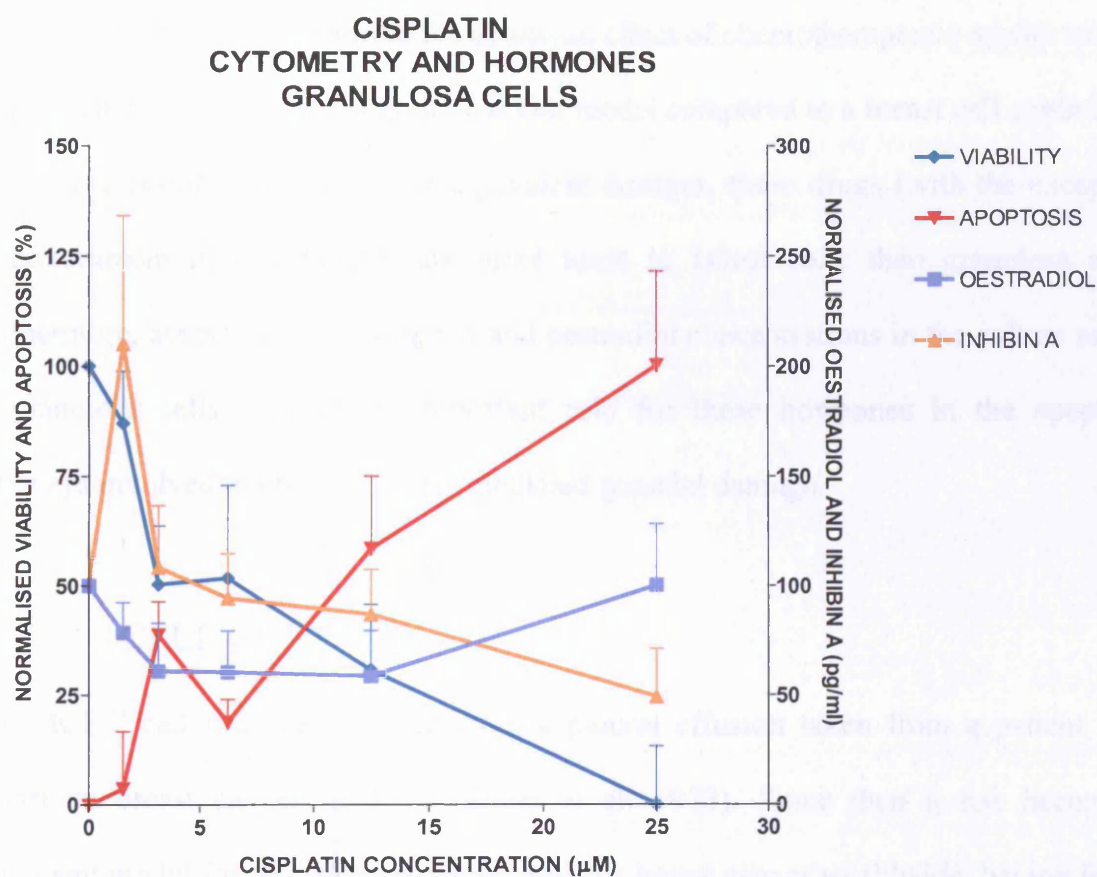


FIGURE 5.5.4 Combined cytometry and hormone data for granulosa cells – cisplatin.
Cytometry and hormone data overlaid using a doubly Y-axis against cisplatin concentration.

5.6 DISCUSSION

5.6.1 INTRODUCTION

This is the first study to examine the cytotoxic effect of chemotherapeutic agents used to treat breast cancer, by using a granulosa cell model compared to a breast cell control.

The results indicate that at equivalent dosages, these drugs (with the exception of doxorubicin in this model) are more toxic to breast cells than granulosa cells. Furthermore, assessment of inhibin A and oestradiol concentrations in the culture media of granulosa cells suggest an important role for these hormones in the apoptotic pathways involved in chemotherapy-mediated gonadal damage.

5.6.2 CELL CULTURE

The MCF-7 cell line was derived from a pleural effusion taken from a patient with metastatic breast cancer in 1970 (Soule et al. 1973). Since then it has become a prominent model for oestrogen receptor-positive breast cancer worldwide, having found many applications in experimental therapeutics (Levenson & Jordan 1997). This cell line has been studied longer than any other in breast cancer, and as a result is very well characterized in the literature. Many variants exist, all of which are considered to be excellent as in vitro models for studying the mechanisms of chemoresistance as it relates to susceptibility to apoptosis (Simstein et al. 2003). Apoptotic pathways for several of the drugs used in this study have been identified to act via the Bcl-2 family of proteins using this system. For example, (4-hydroxy)cyclophosphamide increases pro-apoptotic Bax levels, while doxorubicin causes a decrease in Bcl-2 expression and an increase in Bax levels (Leung & Wang 1999). Paclitaxel has been shown to upregulate several pro-apoptotic Bcl-2 proteins and downregulate anti-apoptotic Bcl-2 proteins (Vakkala et al. 1999).

The use of a cell line in this experiment also provided several other advantages. For instance the cells are relatively robust compared to those obtained from primary culture, and stocks could be easily replenished. There are however several important disadvantages. With successive subcultures, or “passages”, cell lines are prone to genotypic and phenotypic drift (Burdall et al. 2003). In fact, many biological differences, including karyotypic variation, have been shown among MCF-7 cell lines from different laboratories (Bahia et al. 2002; Osborne, Hobbs, & Trent 1987). Overall however breast cell lines, especially those best characterized such as MCF-7, are considered to reflect to a large extent, the features of breast cancer cells in vivo (Lacroix & Leclercq 2004).

Primary granulosa cell culture was chosen as the target cell type for this experiment. In order to study the effects of cytotoxic agents on these cells, it was important to have a well-defined and validated in-vitro culture system available (Muttukrishna, Groome, & Ledger 1997). Granulosa cell death by apoptosis is a critical event in follicular atresia during growth and development of the ovarian follicle (Hughes, Jr. & Gorospe 1991; Tilly et al. 1991). Furthermore, there is evidence that apoptosis-associated signaling pathways are required for chemotherapy-mediated germ cell destruction (Perez et al. 1997). It was logical therefore to study apoptosis as a marker of cytotoxicity shared by both cell types in question.

5.6.3 FLOW CYTOMETRY DATA

The flow cytometric data analysis revealed that the LD50 for all drugs, with the exception of doxorubicin (figure 6.3.1), was lower for breast cells than granulosa cells. For Paclitaxel, the LD50 for granulosa cells was almost forty times that of breast cells (figure 6.3.3), for melphalan almost three times (figure 6.3.2) and for cisplatin more than four times (figure 6.3.4). This implies that these three drugs are far more potent at equivalent doses in breast cells than for granulosa cells. There are some limitations to the interpretation of this data however. In this study, the potency of these drugs is measured in terms of their ability to induce apoptosis in these cells. It is possible that toxicity is exerted by non-apoptotic mechanisms as well. Furthermore, it is not clear what effect these drugs would have when administered as multi-drug combinations, as is the case in vivo. Kugawa and co-workers reported that cell death caused by a combination of cyclophosphamide, doxorubicin and 5-fluorouracil in an MCF-7 cell line was completely non-apoptotic (Kugawa et al. 2004). This stresses the point that while flow cytometric analysis by annexin labeling is very efficient at identifying the frequency of early and late apoptosis in cell populations, it provides limited information on the mechanism of cell death. To achieve this, complementary methods should be used, such as DNA fragmentation analysis (“DNA laddering”), gel electrophoresis of DNA (“COMET”) or end labeling cells to detect DNA strand breaks (TUNEL) (Darzynkiewicz & Traganos 1998). In terms of the aims of this study however, flow cytometry was entirely appropriate.

The implications of the above findings are that it introduces the possibility of tailoring drug treatments that are potent enough to destroy malignant breast cells while sparing granulosa cells, and by extension, preserving ovarian reserve. The need for such

treatments in young women with breast cancer have been mentioned for some time (Goldhirsch et al. 2001).

There are several explanations as to why doxorubicin gave an unexpected result. The most likely is that the MCF-7 line used in our lab had acquired a form of resistance to doxorubicin. At the time the study had started, this particular line had already undergone almost 300 “passages”, raising the possibility that some degree of biological variation might have occurred over time. There are several examples of doxorubicin resistant MCF-7 strains in the literature – engineered or otherwise (Ahn et al. 1996; Brown & Fenselau 2004; Guo-Chang & Chu-Tse 2000). The other possibilities would include inherent problems with establishing the dose response, especially with the granulosa cells, which will be discussed later.

5.6.4 HORMONE ANALYSIS

Luteinised granulosa cells (LGC's) can be functionally assessed by measuring hormone concentrations in the culture medium. These cell culture models have been extensively investigated where secretion of the inhibins, activin and oestradiol have been shown to be regulated by gonadotrophins (Foldesi, Breckwoldt, & Neulen 1998; Lau et al. 1999; Muttukrishna, Groome, & Ledger 1997; Welt & Schneyer 2001). Because inhibin A is considered a product of the mature dominant follicle (and corpus luteum - Groome et al. 1994; Muttukrishna et al. 1994), culture medium from LGC's would be expected to have significant amounts of this hormone.

Our data suggests that at initial concentrations of drug, inhibin A levels are increased significantly with a corresponding decrease in oestradiol levels. There was a consistent pattern of hormone release for all drugs with the exception of Paclitaxel (figures 6.4.1 to 6.4.8). In the case of doxorubicin, melphalan and cisplatin, inhibin A

levels rose two to three fold at the lowest concentration of drug compared to the controls. These levels then gradually recovered to concentrations similar to the control sample. The opposite effect appeared to happen with oestradiol. While not as dramatic compared to inhibin A, there was a clear decline in oestradiol levels following administration of the lowest concentration of drug. These levels rose gradually as drug concentrations increased. These results may be reflective of the apoptotic processes involved. The increased inhibin A levels may reflect a physiologic protective mechanism of the granulosa cell to reduce apoptosis. Granulosa cells (isolated from follicles of women participating in an IVF program) cultured in an enriched inhibin A environment have been shown to correlate with reduced levels of apoptosis (increased anti-apoptotic and reduced pro-apoptotic proteins). This reduction in apoptosis also correlated with increased levels of oestradiol, but the anti-apoptotic effect of inhibin A was still apparent even at low oestrogen levels (Denkova et al. 2004). Other investigators have identified increased oestrogen as being an important factor in reducing apoptosis, possibly mediated by increased inhibin levels (Billig, Furuta, & Hsueh 1993; Campbell & Baird 2001; Song & Santen 2003). This has not been a universal finding however, as some investigators have shown evidence of inhibin A having a negative autocrine or paracrine effect on estradiol production by granulosa cells using a rat culture model (Jimenez-Krassel et al. 2003). Others have shown that inhibin A increases apoptosis in early ovarian antral follicles of rats (Vitale et al. 2002).

Another explanation for increased inhibin A levels is that inhibiting FSH release from the anterior pituitary is a physiological mechanism to reduce oestradiol secretion by granulosa cells (Ho et al. 2006). Oestradiol secretion by granulosa cells was not measured in that study, limiting adequate elucidation of the proposed mechanism. It may be possible however that increased inhibin levels as a result of cytotoxic injury contributes to the hypo-oestrogenic state seen in patients following chemotherapy.

While our results indicate that basal oestradiol levels are reduced, we could not find any significant increase in inhibin A levels compared to the controls. This may be explained by the fact that this was an *in vitro* study, and as such, the physiologic effect of FSH suppression would not be a factor. The question remains whether inhibin A exerts its influence on oestradiol release via the HPO axis, or in an autocrine/paracrine fashion at the level of the ovary, or a combination of both. Cytotoxic agents used to treat breast cancer may induce these changes by interacting with membrane-signalling pathways on granulosa cells. Further study in this area would involve using a narrower dose range to pinpoint the events which occur at the time of inhibin release. Additionally, culture conditions might be manipulated further in an effort to mimic the physiologic conditions seen in humans.

The only other study which experimented on granulosa cells *in vitro* measured progesterone concentration in cultured rat and human granulosa cells exposed to 4-hydroperoxy (activated) cyclophosphamide *in vitro* (Ataya, Pydyn, & Ramahi-Ataya 1990). This study limited its analysis to a single agent and no comparison with the effect on breast cells was attempted.

This study has provided useful insight into the dose-related response of granulosa cells to cytotoxic agents used to treat breast cancer. Based on these results, it seems plausible to investigate further the possibility of modifying dosages of cytotoxic agents so that a lethal effect is incurred on malignant breast cells while sparing that of granulosa cells. Further research is needed regarding the mechanism of cell kill in granulosa cells however. One option is to perform COMET assays on both cell types to determine which cell type has more efficient DNA repair to determine which cell type has a more efficient DNA repair apparatus.

6. GENERAL DISCUSSION

6.1 IN VIVO STUDY

The results of this study show that biochemical and biophysical tests may be used to accurately estimate ovarian reserve in premenopausal recipients of chemotherapy for breast cancer. This can have a major impact on the management of fertility-related issues and premature ovarian failure in these patients.

Markers of ovarian reserve have been assessed for some time in the fertility setting, where it became established that clinical characteristics alone were unreliable in predicting reproductive age. So far, biochemical and biophysical parameters appear to be more reliable than chronological age. Despite extensive investigation however, the ideal test remains to be identified. To the best of our knowledge, no definitive single marker for assessing ovarian reserve in patients with cancer has been identified, leading to the use of multiple markers (Lutchman Singh, Davies, & Chatterjee 2005).

In general, patients in this study all received cyclophosphamide-based chemotherapy. All patients resumed regular menstrual cycles after a variable period of amenorrhoea indicating that the effects of these agents are at least partially reversible. Despite this, clinical characteristics (patient age and BMI) were not found to be discriminatory once cyclical activity had resumed. This is in keeping with the generally accepted notion that clinical characteristics alone are not reliable estimates of reproductive age. Biochemical markers, which appeared to be discriminatory, included FSH, AMH, inhibin B and oestradiol in response to the G-test. The only discriminatory biophysical marker was the antral follicle count. There was good correlation between AFC and other markers of ovarian reserve. These results taken together support the hypothesis that ovarian reserve is reduced in young, regularly cycling women following treatment with chemotherapeutic agents for breast cancer. The data also add to the

understanding of the pathophysiological processes involved, in that ovarian reserve was potentially intact in these patients prior to chemotherapy. This implies that chemotherapy was the main factor that led to diminished ovarian reserve.

Young women treated for cancer have unmet needs for fertility and menopause-related information (Duffy, Allen, & Clark 2005). Counselling about the effect of chemotherapy on premature ovarian failure and fertility is also considered an overlooked aspect of preparation for adjuvant chemotherapy in young women with breast cancer (Thewes et al. 2005). Many patients are left with significant anxieties and insufficient information about reproductive issues (Schover et al. 1999). A unique web-based survey of fertility issues in young women with breast cancer revealed that only 51% felt that their concerns were addressed adequately. Also, 29% percent of women reported that infertility concerns influenced treatment decisions (Partridge et al. 2004).

Estimation of functional ovarian reserve in these patients can have a significant impact on how patients are counselled both before and after chemotherapy. These tests can be applied both pre and post-chemotherapy where patients can be classified into 3 groups: normal, poor and intermediate ovarian reserve. In the pre-chemotherapy patient, this information can be used to predict the response to ovarian stimulation for embryo/egg cryopreservation, allowing appropriate counselling and modification of treatment. For example patients deemed to have normal ovarian reserve prior to potentially sterilising chemotherapy might opt to have embryos cryopreserved knowing that their response to COH is likely to be adequate. The same may be applied to patients post-chemotherapy for treatment of subfertility. Alternatively, patients with normal ovarian reserve may be counselled regarding adequate contraception. Furthermore, the potential exists to counsel patients appropriately regarding the onset of a premature menopause. In some cases, treatment decisions for the patient's cancer can be affected. For example in women with breast cancer where there is an option for either cytotoxic

chemotherapy or reversible endocrine ablation, the latter may be preferred if ovarian reserve is already diminished prior to treatment.

This study was limited by small sample size and limited longitudinal analysis (for reasons described earlier). Despite this, it is important in several respects. It is the first of its kind to evaluate dynamic ovarian reserve in a young, cycling breast cancer cohort, all of whom had received cyclophosphamide-based chemotherapy. The methodology employed is robust and included elaborate methods of testing— all of which appeared to be well tolerated. To realise the full potential for ovarian reserve testing in these patients, longitudinal follow up with a larger number of patients would be important to investigate the recovery of ovarian function in these young women with breast cancer, and allow stratification according to chemotherapy type and dose. To achieve this, large, prospective, adequately controlled studies are required specific to different geographical areas (multi-centre) in a control population of comparable reproductive age to determine the potential role of ovarian reserve testing in clinical practice. This would allow specific data such as pregnancy outcome and age at menopause to be acquired, enabling correlation with markers of ovarian reserve. The aim would be to develop a clinical tool, which may allow classification of ovarian reserve (e.g. into normal, intermediate, and poor). This tool would allow a more accurate assessment of ovarian reserve including the prediction of response to future treatment.

Furthermore, should multi-agent chemotherapy prove to be the main factor affecting ovarian reserve, the potential exists for ovarian reserve testing to be applied to different cancer cohorts.

One must stress however that these examples represent only the *potential* of ovarian reserve testing in these patients, as these applications remain as yet unproven. Furthermore, while fertility and quality of life issues are important, they must not be pursued at the expense of the patient's overall treatment and welfare. While potentially informative, ovarian reserve testing can only help and clarify the decision-making process in a limited group of patients, as malignancy and reproductive issues are complex and difficult.

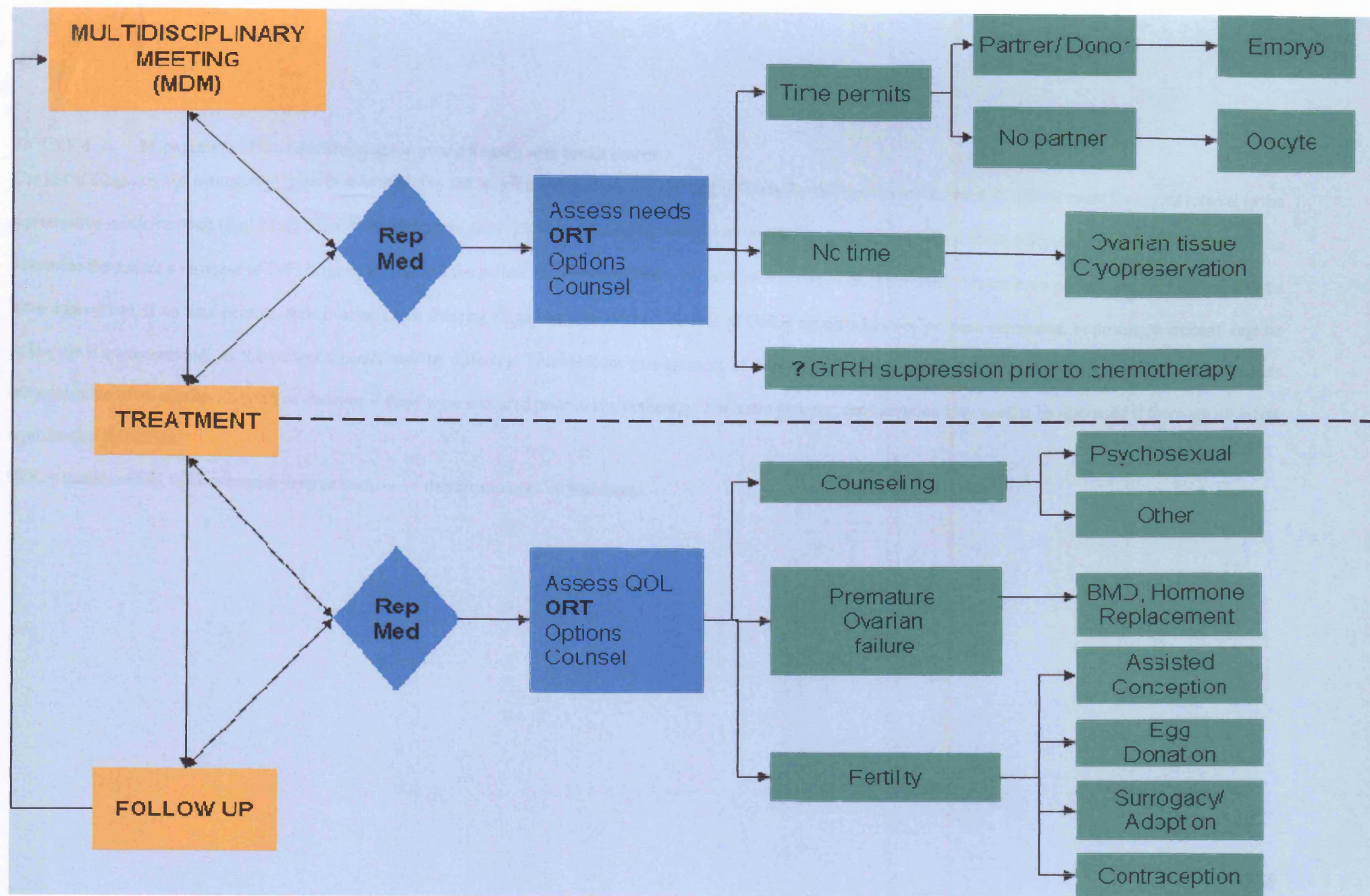


FIGURE 6. 1 Management of reproductive issues in young women with breast cancer.

The initial diagnosis and management plan is determined by the breast multi-disciplinary meeting (MDM). By this point, consideration should be made for urgent referral to the reproductive medicine team (Rep Med). Here, ovarian reserve tests might be performed, which can be used to counsel and treat patients effectively especially if the aim is to determine the patient's response to IVF. If time permits, and the patient has a partner, IVF with embryo freezing is recommended. If there is no partner, oocyte freezing may be more appropriate. If no time permits, then ovarian tissue freezing should be considered. The role of GnRH agonists has not yet been elucidated. Following treatment, regular follow-up is recommended, as the patient's needs may be different. This includes management of POF-related effects and restoration of fertility. The latter may include reimplantation of an ovarian allograft or embryos if these were procured prior to chemotherapy. For some patients, contraception may need to be discussed if there are no active reproductive ambitions.

QOL = quality of life, ORT = ovarian reserve tests, ---- = demarcation pre vs postchemo.

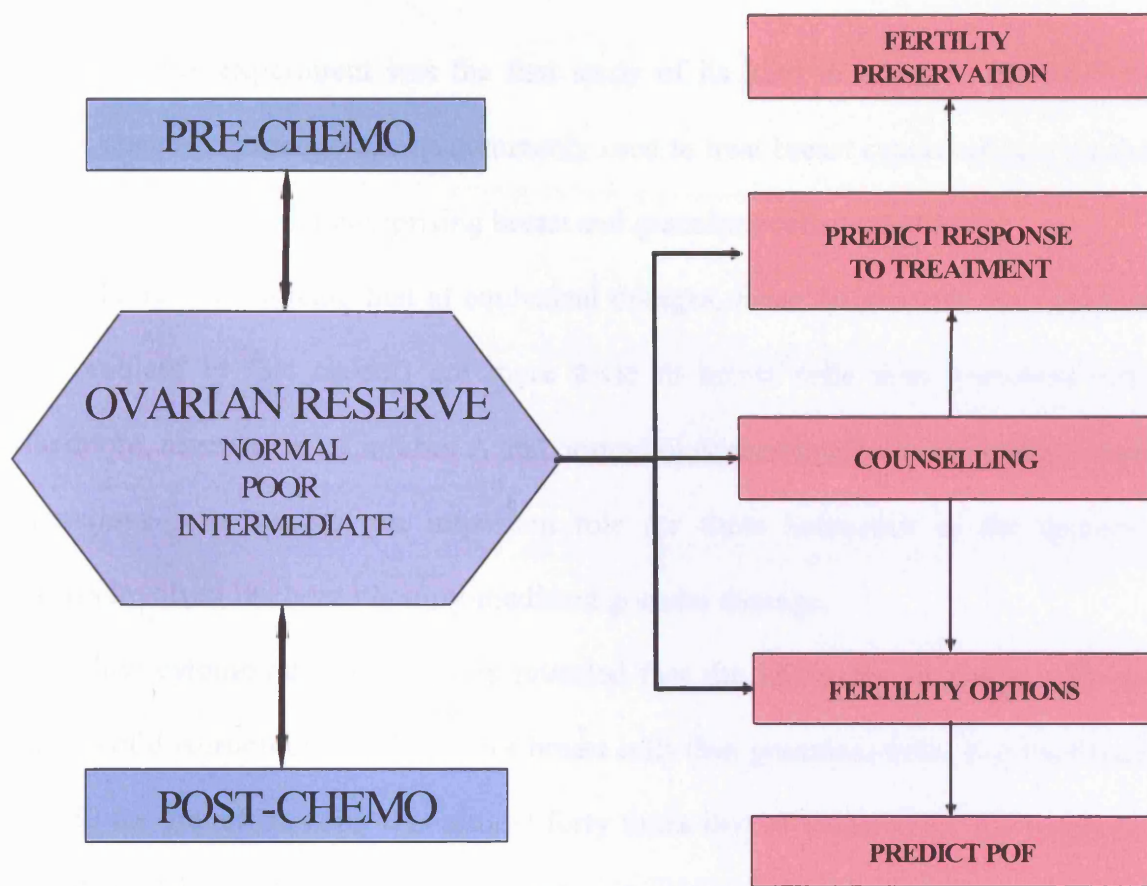


FIGURE 6. 2 The proposed role of ovarian reserve tests in young women with breast cancer.

ORT can be applied pre- and post-chemotherapy to classify patients into 3 groups – normal, poor and intermediate ovarian reserve as a guide to counseling and treatment respectively.

6.2 IN VITRO STUDY

The cell culture experiment was the first study of its kind to compare the cytotoxic effect of chemotherapeutic agents commonly used to treat breast cancer using a parallel invitro cell culture model comprising breast and granulosa cells respectively.

The results indicate that at equivalent dosages, these drugs (with the exception of doxorubicin in this model) are more toxic to breast cells than granulosa cells. Furthermore, assessment of inhibin A and oestradiol concentrations in the culture media of granulosa cells suggest an important role for these hormones in the apoptotic pathways involved in chemotherapy-mediated gonadal damage.

Flow cytometric data analysis revealed that the LD50 for all drugs, with the exception of doxorubicin, was lower for breast cells than granulosa cells. For Paclitaxel, the LD50 for granulosa cells was almost forty times that of breast cells, for melphalan almost three times and for cisplatin more than four times. This implies that these three drugs are far more potent at equivalent doses in breast cells than for granulosa cells. There are some limitations to the interpretation of this data however. In this study, the potency of these drugs is measured in terms of their ability to induce apoptosis in these cells. It is possible that toxicity is exerted by non-apoptotic mechanisms as well. For this reason, future experiments would need to incorporate tests which elucidate the mechanism of cell death, such as DNA fragmentation analysis ("DNA laddering"), gel electrophoresis of DNA ("COMET") or end labeling cells to detect DNA strand breaks (TUNEL). Another point for consideration in the future is to employ multi-drug administration (analogous to in vivo use), as it is not clear what effect this would have.

One explanation for the fact that doxorubicin's results were not similar to the other drugs is that this was a true effect – that is doxorubicin is in fact more toxic to granulosa cells than breast cells. This would seem unlikely given the fact that alkylating

agents appear to have a greater cytotoxic effect on ovarian reserve in vivo compared to anthracycline drugs. One would not expect therefore, that doxorubicin would be more toxic to granulosa cells than melphalan when compared to breast cells. What is more likely is the development of doxorubicin resistance to the MCF-7 cell line in vitro.

One of the main limitations of this experiment was that the cell culture conditions were not fully optimised. This pertained especially with regards to the granulosa cell culture. In comparison, the breast cell culture was relatively easy to perform and sustain as would be expected with cell line (an advantage of using this resource). The primary granulosa cell culture was particularly difficult to maintain beyond 48 hours. One of the main reasons for this could be contamination of the specimen at the time of collection (ie. at the time of follicular aspiration). Ideally cell culture conditions would have been optimised to achieve sufficient cell viability to 72 hours as was the case with the breast cell culture. It would be imperative that cell culture conditions are optimised prior to any future experiments of this nature.

The release of inhibin A and oestradiol into the culture medium by the granulosa cells may provide a further means of elucidating the mechanism of cell death in granulosa cells exposed to cytotoxic agents. It is possible that cytotoxic agents used to treat breast cancer may induce these changes by interacting with membrane-signalling pathways on granulosa cells. Further study in this area would involve using a narrower dose range to pinpoint the events which occur at the time of inhibin release. Progesterone levels could be included in the analysis as this is also expressed by luteinised granulosa cells in vitro. It would be instructive to assess the effect of cytotoxic agents on hormone release of breast cells in vitro. The possibilities here could include activin A and Inhibin A. If these hormones were released from breast cells in a functional manner analogous to granulosa cells, it might be possible to calculate dose

response curves on both cell types after administration of cytotoxic agents and compare them with dose obtained from flow cytometry to determine if any correlation existed.

The implications of the above findings are that it introduces the possibility of tailoring drug treatments which are potent enough to destroy malignant breast cells while sparing granulosa cells, and by extension, preserving ovarian reserve. If the mechanism of granulosa cell cytotoxicity could be elucidated, it may be possible to choose cytotoxic agents based on this knowledge. This experiment could also be modified to examine the effect of agents that suppress ovarian apoptosis (sphingosine-1-phosphate) as has been shown previously in mice (Morita et al. 2000). Further research is needed regarding the mechanism of cell kill in granulosa cells however. One option is to perform COMET assays on both cell types to determine which cell type has more efficient DNA repair to determine which cell type has a more efficient DNA repair apparatus.

Finally, this experiment could be incorporated into the clinical study discussed in section 6.1. For example, patients undergoing fertility preservation prior to chemotherapy could have follicular fluid samples obtained at the time of embryo/egg capture and the resulting granulosa cell culture used to perform the same experiments described, using the same chemotherapy combination administered in vivo. This would allow a more accurate assessment of the cytotoxic effect of these agents on granulosa cells, and correlate it with markers of ovarian reserve. The control sample would be obtained from patients undergoing controlled ovarian hyperstimulation for unexplained or male factor infertility.

6.3 CONCLUSION

Addressing the quality of life concerns of young patients with breast cancer has assumed ever increasing importance due to the progressive rise in incidence rates coupled with improved survival. There is a lack of pertinent data relating to ovarian reserve testing in breast cancer, and information pertaining to ORT in a subfertile population cannot be extrapolated to include patients with breast cancer, as the pathophysiology is distinct.

This thesis has contributed to the establishment of potentially useful biochemical and biophysical parameters of ovarian reserve in young women with breast cancer. Further research could potentially allow ovarian reserve testing to be applied clinically in patients with cancer as a guide to counselling and treatment in terms of fertility preservation.

The in vitro study was performed to supplement the in vivo study. This provided evidence to support the notion that cytotoxic agents used to treat breast cancer might have varying degrees of toxicity on malignant breast cells compared to granulosa cells as measured by apoptosis. Further study in this area might eventually lead to correlation with clinical outcome in vivo. The aim would be to develop drugs which are selectively cytotoxic to malignant breast cells, whilst having minimal or no effect on granulosa cells.

Finally, the objective of this information would always be for the benefit of the psychological and physical well being of the patient.

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8. APPENDICES

8.1 PUBLICATION 1

Lutchman Singh, K., Davies, M., & Chatterjee, R. 2005, "Fertility in female cancer survivors: pathophysiology, preservation and the role of ovarian reserve testing", *Hum.Reprod.Update.*, vol. 11, no. 1, pp. 69-89.

8.2 PUBLICATION 2

Lutchman, S. K., Muttukrishna, S., Stein, R. C., McGarrigle, H. H., Patel, A., Parikh, B., Groome, N. P., Davies, M. C., & Chatterjee, R. 2007, "Predictors of ovarian reserve in young women with breast cancer", *Br.J.Cancer*, vol. 96, no. 12, pp. 1808-1816.

8.3 INFORMATION LEAFLETS FOR IN VIVO STUDY

8.4 CONSENT FORMS FOR IN VIVO STUDY

Fertility in female cancer survivors: pathophysiology, preservation and the role of ovarian reserve testing

Kerryn Lutchman Singh¹, Melanie Davies and Ratna Chatterjee

K.Lutchman Singh, M.Davies and R.Chatterjee

Fertility in female cancer survivors

K.Lutchman Singh, M.Davies and R.Chatterjee

Fertility in female cancer survivors

K.Lutchman Singh, M.Davies and R.Chatterjee

Fertility in female cancer survivors

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Fertility in female cancer survivors

K.Lutchman Singh, M.Davies and R.Chatterjee

Fertility in female cancer survivors

K.Lutchman Singh, M.Davies and R.Chatterjee



Predictors of ovarian reserve in young women with breast cancer

**K Lutchman Singh^{*,1}, S Muttukrishna¹, RC Stein², HH McGarrigle¹, A Patel¹, B Parikh¹, NP Groome³,
MC Davies¹ and R Chatterjee¹**



Ovarian reserve predictors in young women with breast cancer

K Lutchman Singh *et al*



Ovarian reserve predictors in young women with breast cancer

K Lutchman Singh *et al*



Ovarian reserve predictors in young women with breast cancer

K Lutchman Singh *et al*





CONFIDENTIAL

INFORMATION LEAFLET (Volunteers)

Estimation of ovarian reserve in patients treated by cytotoxic drugs for cancer

We would like to ask you to take part in the above-mentioned study.

What is this study about?

Chemotherapy used for treatment is likely to cause damage to the ovaries of someone treated for cancer. This may affect their ability to produce eggs properly, which can lead to problems with having children (fertility), affect their periods and can even lead to an early menopause. Not everyone is affected in the same way as patients may still have regular periods and have the ability to produce eggs (ovulation) and also have children. However, having regular periods does not guarantee that the ovaries are producing eggs normally.

Currently no reliable methods are available to find out which patients treated by chemotherapy can produce eggs normally. With early diagnosis and better cancer care, many patients are now surviving longer and would like to have a family.

We are a medical team based at University College Hospital who is interested in performing a study, which looks at whether or not we can use certain tests to tell if chemotherapy received during treatment for cancer caused damage to the ovaries. We plan to do this by working out something called "ovarian reserve". This is basically is the amount of "good" eggs that the ovary contains. This study is primarily aimed to help cancer patients. Although we hope that the information we obtain from this study can be used to advise and help them regards having a family, we must tell you that any information we get from your taking part in this study would not be of any direct benefit to yourself.

What will I be asked to do?

Basically there are two tests we would like you to have, both of which are essentially harmless and cause little or no pain. This includes blood tests and ultrasound scan of the pelvis, which will be done at two stages, with and without an injection of a hormone called buserelin.



Test without injection:

On the second day of your period we would like you to come in to hospital clinic. We would then take your medical history like we would in a normal clinic visit. After this, we would need to take some blood tests over a 1-hour period. This would be done by placing a single needle in your arm with an attachment, so we don't need to inject you more than once. While the blood test is being done, we would like to perform an ultrasound looking at your ovaries in particular. Usually this is done by placing the scan on your tummy, but sometimes if we can't see your ovaries properly, we do so by placing the scan in the vagina. This is called a transvaginal scan, and feels similar to having a tampon inserted.

Test with injection:

The last part of the test is called the G-test. To do this, we have to give you an injection under the skin containing a drug called buserelin. This drug works by causing your ovaries to release oestrogen hormone, in almost the same way that your brain releases hormones to cause the ovary to work properly. After this is given, we would need you to come back at the same time the next day to have one final blood test taken.

How often must these tests be done?

We would like you to have the test only once

Are there side effects with these tests?

Blood tests are routine tests you may have had before for other reasons. This is exactly the same. Sometimes it can cause bruises and some discomfort. USS is usually painless apart from the pressure sensation and the discomfort or messiness of the jelly, which is used during scanning.

Are there side effects with taking buserelin?

Sometimes women who take this drug for a long period experience symptoms like the menopause such as hot flushes. We do not believe this will happen in your case because we are only giving you one dose.

Are there any safety measures I should take before doing the test?

Yes. If you were taking the oral contraceptive pill, we would need you to stop taking them for 1 month before the tests are performed. Remember to use barrier methods of family planning such as condoms or a diaphragm in the meantime.



What happens to the information you acquire?

Any information we obtain from your history or investigations during the study will be kept strictly confidential and managed by UCLH. Your name and address will be kept separately from any information about you so that you cannot be identified from it. The members of the research team will be responsible for all information related to the study.

What happens to the samples that are taken?

All the samples are usually stored in a freezer until they are tested. We usually store these samples for some time (5 years or more). This allows us to repeat tests on the same samples later on, such as when a new test is discovered. Eventually samples are destroyed by fire.

Do I have to take part?

It is up to you to decide whether or not to take part in this study. If you decide to take part you are free to change your mind at any time and without giving a reason.

What if I have questions?

You must feel free to contact any member of the study group to answer any questions you might have. You can contact us by post or phone or e-mail Dr Lutchman Singh at his e-mail address. If you contact us by phone we will be happy to phone you back. We will be happy to answer your questions.

THANK YOU FOR CONSIDERING TAKING PART IN THIS STUDY

Research team:

Miss Melanie Davies (principal investigator)

Dr Ratna Chatterjee

Dr Kerry Lutchman Singh

Contact details:

Address: Reproductive Medicine Unit

Tel:

e-mail:



CONFIDENTIAL

PATIENT INFORMATION LEAFLET

Estimation of ovarian reserve in patients treated by cytotoxic drugs for cancer

We would like to ask you to take part in the above-mentioned study.

What is this study about?

We know that chemotherapy you received (or will receive) to treat your cancer is likely to cause damage to your ovaries. This may affect your ability to produce eggs properly, which can lead to problems with having children (fertility), affect your periods and can even lead to an early menopause. Not everyone is affected in the same way, as some of you may still have regular periods and have the ability to produce eggs (ovulation) and also have children. However, having regular periods does not guarantee that your ovaries are producing eggs normally.

Currently no reliable methods are available to find out which patients treated by chemotherapy can produce eggs normally. With early diagnosis and better cancer care, many patients are now surviving longer and would like to have a family.

We are a medical team based at University College Hospital (UCLH) who are interested in performing a study which looks at whether or not we can use certain tests to tell us if chemotherapy you received during your treatment for cancer have damaged your ovaries. We plan to do this by working out your ovarian reserve, which basically is the amount of "good" eggs you have in your ovary. We think it is important that you know this information so that you may be better equipped to plan your future regarding children. This information would also be useful to your doctors if you ever needed help to have children. We hope eventually to be able provide this information to all women who have been treated for cancer with different types of chemotherapy.

What will I be asked to do?

Basically there are two tests we would like you to have, both of which are essentially harmless and cause little or no pain. This includes blood tests and ultrasound scan of the pelvis, which will be done at two stages, with and without an injection of a hormone called buserelin.



Test without injection:

On the second day of your period we would like you to come in to hospital clinic. We would then take your medical history like we would in a normal clinic visit. After this, we would need to take some blood tests over a 1-hour period. This would be done by placing a single needle in your arm with an attachment, so we don't need to inject you more than once. While the blood test is being done, we would like to perform an ultrasound looking at your ovaries in particular. Usually this is done by placing the scan on your tummy, but sometimes if we can't see your ovaries properly, we do so by placing the scan in the vagina. This is called a transvaginal scan, and feels similar to having a tampon inserted.

Test with injection:

The last part of the test is called the G-test. To do this, we have to give you an injection under the skin containing a drug called buserelin. This drug works by causing your ovaries to release oestrogen hormone, in almost the same way that your brain releases hormones to cause the ovary to work properly. After this is given, we would need you to come back at the same time the next day to have one final blood test taken.

How often must these tests be done?

We would like you to have the test 5 times: before you have the chemotherapy, immediately after completion of the chemotherapy, at 6 months, 12 months and at 24 months after completion of chemotherapy. If you have already finished your chemotherapy, then the first test will be omitted.

Are there side effects with these tests?

Blood tests are routine tests you must be having for your cancer treatment. Sometimes it can cause bruises and some discomfort. USS is usually painless apart from the pressure sensation and the discomfort or messiness of the jelly, which is used during scanning.

Are there side effects with taking buserelin?

Sometimes women who take this drug for a long period experience symptoms like the menopause such as hot flushes. We do not believe this will happen in your case because we are only giving you one dose.

Are there any safety measures I should take before doing the test?

Yes. If you were taking the oral contraceptive pill (OCP), we would need you to stop taking them for 1 month before the tests are performed. Remember to use barrier methods of family planning such as condoms or diaphragm in the meantime.



What happens to the information you acquire?

Any information we obtain from your history or investigations during the study will be kept strictly confidential and managed by UCLH. Your name and address will be kept separately from any information about you so that you cannot be identified from it. The members of the research team will be responsible for all information related to the study.

What happens to the samples that are taken?

All the samples are usually stored in a freezer until they are tested. We usually store these samples for some time (5 years or more). This allows us to repeat tests on the same samples later on, such as when a new test is discovered. Eventually samples are destroyed by fire.

Do I have to take part?

It is up to you to decide whether or not to take part in this study. If you decide to take part you are free to change your mind at any time and without giving a reason. Whatever your decision, it will not affect the standard care that you receive in any way.

What if I have questions?

You must feel free to contact any member of the study group to answer any questions you might have. You can contact us by post or phone or e-mail Dr Lutchman Singh at his e-mail address. If you contact us by phone we will be happy to phone you back. We will be happy to answer your questions.

THANK YOU FOR CONSIDERING TAKING PART IN THIS STUDY

Research team:

Miss Melanie Davies (principal investigator)

Dr Ratna Chatterjee

Dr Kerry Lutchman Singh

Contact details:

Address: Reproductive Medicine Unit

Tel:

e-mail:



CONSENT FORM (Volunteers)

THIS COPY OF THE CONSENT FORM IS FOR YOU TO KEEP

Patient identification number for study:

Form version:

Name of principal researcher: Miss Melanie Davies

Please initial box

I confirm that I have read and understood the information sheet dated
(version) for the above study and have had the opportunity to ask questions.

I confirm that I have had sufficient time to consider whether or not I want to be
included in the study

I understand that my participation is voluntary and that I am free to withdraw
at any time and without giving any reason.

I agree to take part in the above study.



Title of project: Estimation of ovarian reserve in patients treated by cytotoxic drugs for cancer

Name of Principal investigator : Miss Melanie Davies

Name of volunteer

Date

Signature

Name of Person taking consent
(if different from researcher)

Date

Signature

Name of the researcher to contact if there are any problems



CONSENT FORM (Patients)

THIS COPY OF THE CONSENT FORM IS FOR YOU TO KEEP

Patient identification number for study:

Form version:

Name of principal researcher: Miss Melanie Davies

Please initial box

I confirm that I have read and understood the information sheet dated
(version) for the above study and have had the opportunity to ask questions.

I confirm that I have had sufficient time to consider whether or not I want to be
included in the study

I understand that my participation is voluntary and that I am free to withdraw
at any time, without giving any reason, without my medical care or legal rights
being affected.

I understand that sections of any of my medical notes may be looked at by members
from the research team, or from regulatory authorities where it is relevant to my taking
part in research.

I give permission for these individuals to have access to my records.

I agree to take part in the above study.

I agree / do not agree (please delete as appropriate) for my sibling(s) to be approached
to take part in this study.

I understand that I am under no obligation to allow my sibling(s) to be approached and
I am free to change my mind at any time without my medical care or participation in this
study being affected



Title of project: Estimation of ovarian reserve in patients treated by cytotoxic drugs for cancer

Name of Principal investigator : Miss Melanie Davies

Name of patient

Date

Signature

Name of Person taking consent
(if different from researcher)

Date

Signature

Name of the researcher to contact if there are any problems



CONSENT TO DISCLOSURE OF CLINICALLY RELEVANT INFORMATION

It has been fully explained to me that the results of the tests that I have had performed as part of this study may be relevant to my clinical care. I understand fully the usefulness and limitations of this information.

I would like to consent to the disclosure of clinically relevant data in the following manner:
(delete as appropriate)

My oncologist or consultant dealing with my treatment: Yes/No/Undecided

My GP: Yes/No/Undecided

I consent to the inclusion of clinically relevant data into my medical case notes where any member of the medical team responsible for my care may have access to it as part of my clinical care:

Yes/No/Undecided

PATIENT NAME:

SIGNATURE:

DATE:

8.5 LABORATORY EQUIPMENT AND CONSUMABLES

8.5.1 GENERAL EQUIPMENT IN VIVO STUDY

ITEM	MANUFACTURER/SUPPLIER
Automated Plate Washer	Wellwash 4 Mk II plate washer (Thermo Electron Corp, Bioscience Technologies, Basingstoke, UK)
MRX 2 Microplate Reader	Dynex Technologies, Chantilly, VA, USA.
Nunc-Immuno™ Maxi Sorp 96 MicroWell™ Plates	NuncBrand, Fisher Scientific UK Loughborough, Leicestershire LE11 5RG, UK.
Plate shaker	Labsystems Wellmix, Finland
Shaking waterbath-CS 200 G refrigerated immersion cooler	Grant Instruments (Cambridge) Ltd., Cambridgeshire, UK.
Whorly mixer (Vortex- Genie 2)	Scientific Industries, Inc. Bohemia, NY 11716 USA.

8.5.2 GENERAL EQUIPMENT IN VITRO STUDY

NAME OF APPARATUS	SUPPLIER/MAKE
AUTOCLAVE Midas 40 priorclave	Priorclave Ltd 129/131 Nathan Way Woolwich SE28 0AB, UK.
DISPENSERS MANUAL MULTICHANNEL	Scientific laboratory supplies (SLS) Wilford Industrial Estate Nottingham, NG11 7EP, UK.
DISPENSERS ELECTRONIC MULTICHANNEL (Genex Alpha)	Genex Unit 1, Barton Hill Way, Torquay TQ2 8JG UK.
DISPENSERS SEMI-AUTOMATED PIPETTE	Gilson, S.A.S, 95400 vlliers le Bel, France
DISPENSER ELECTRONIC PIPETTE GUN (Genex Delta)	Genex Delta Unit 1, Barton Hill Way, Torquay TQ2 8JG UK.
ELECTRONIC SCALE	A & D instruments, Abingdon, Oxford, UK
FLOW CYTOMETER + SOFTWARE Beckman-Coulter EPICS ELITE Software- B/C Flow station analysis version 4.5	Beckman- Coulter, Miami, FL, U.S.A
FREEZER (- 20° C)	Liebherr, Germany
HEMOCYTOMETER	SIGMA Chemical Co; St Louis, USA
HUMIDIFIED INCUBATOR	LEEC Ltd Nottingham, UK

LAMINAR FLOW CABINET	Heto-Holten (UK) Ltd, Camberley, Surrey, UK Gelaire flow laboratories, North Somerset, UK
LARGE CENTRIFUGE (Centaur 2 bench-top centrifuge)	Fisons scientific (MSE) instruments Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.
MAGNETIC STIRRER	Jencons-PLS Unit 15, Imberhorne Industrial Estate East Grinstead, West Sussex, RH19 1XZ UK.
MINI-CENTRIFUGE	Sanyo Gallenkamp PLC, Loughborough, Leics, UK
MULTIFUNCTION TOPLOADING ANALYTICAL BALANCES	A& D instruments, Abingdon, Oxford, UK.
OPTICAL MICROSCOPE (CK30/ CK40 culture microscope)	Olympus Optical Co., Ltd., Tokyo, Japan
pH METER	Jencons-PLS Unit 15, Imberhorne Industrial Estate West Sussex, RH19 1XZ, UK.

8.5.3 CONSUMABLES (IN VIVO AND IN VITRO)

ITEM	MANUFACTURER OR SUPPLIER
2 ml FLAT BOTTOM TUBES WITH CAPS	Hughes and Hughes Ltd, UK.
5, 10,25,50 ML STERILE CONTAINERS	Bibby Sterilin Ltd, Staffordshire, UK.
STERILE AND NON –STERILE PLASTIC PASTEUR PIPETTES	Alpha laboratories Ltd, Hampshire, UK.
5, 10, 25ml STERILE PIPETTES	Sterilin, Bibby sterilin Ltd, Staffordshire, UK.
20, 200 AND 1000 μ L PIPETTE TIPS (NON- STERILE) (Manufacturer – Gilson; Calibration – Caltech)	Gilson, S.A.S, 95400 vlliers le Bel, France. Caltech 4 Tanners Drive, Milton Keynes MK14 5NA, UK.
CYTOMETER TUBES (FOR PLACING SAMPLES IN CYTOMETER)	BD Falcon, BD Biosciences Europe, Belgium
EPPENDORF TUBES	Sarstedt, Aktlengesellschaft & Co., Germany
96-WELL MICROTITER PLATES	NuncBrand, Fisher Scientific Loughborough, Leicestershire LE11 5RGUK.
STERILE 24-WELL CULTURE DISHES WITH LIDS	NuncBrand, Fisher Scientific Loughborough, Leicestershire LE11 5RGUK.

STERILE 24 AND 80CM ² CULTURE FLASKS	NuncBrand, Fisher Scientific UK Loughborough, Leicestershire LE11 5RG UK.
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8.5.4 REAGENTS (IN VIVO STUDY)

ITEM	MANUFACTURER/SUPPLIER
AMH ASSAY KIT	Immunotech SA Marseille, France.
INHIBIN B ASSAY KIT DSL-10-84100 ACTIVE® Inhibin B Enzyme- Linked Immunosorbent (ELISA)	Diagnostic Systems laboratories, Inc. Texas, USA.
FSH ASSAY KIT	IBL Immuno-Biological Laboratories Hamburg, Germany.
LH ASSAY KIT	IBL Immuno-Biological Laboratories Hamburg, Germany.
17 beta-Estradiol KIT	IBL Immuno-Biological Laboratories Hamburg, Germany.
ELISA AMPLIFICATION SYSTEM	Invitrogen Life Technologies Paisley, UK.
BOVINE SERUM ALBUMIN (BSA)	Sigma-Aldrich Company Ltd. Gillingham, Dorset SP8 4XT, UK.
CONCENTRATED HYDROCHLORIC AND SULPHURIC ACID	Sigma-Aldrich Company Ltd. Gillingham, Dorset SP8 4XT, UK.
MOUSE SERUM	Sigma-Aldrich Company Ltd. Gillingham, Dorset SP8 4XT, UK.
Triton® X-100	Sigma-Aldrich Company Ltd. Gillingham, Dorset SP8 4XT, UK.

8.5.5 REAGENTS (IN VITRO STUDY)

REAGENT	MANUFACTURER/SUPPLIER
ANNEXIN	Roche Diagnostics Ltd. Bell Lane, Lewes East Sussex, BN 7 1LG UK.
AMPHOTERICIN	GIBCO cell culture; Invitrogen Ltd , Paisley, UK
DMSO	Sigma- Aldrich Company Ltd. St Louis., USA
DMEM/F12 MEDIUM	GIBCO cell culture; Invitrogen Ltd , Paisley, UK.
EDTA 0.02 % in PBS	PromoCell GmbH Sickingenstraße Heidelberg, Germany.
FORMALIN (37% in solution)	Sigma-Aldrich Company Ltd. Gillingham, Dorset SP8 4XT, UK.
GENTAMYCIN	GIBCO cell culture; Invitrogen Ltd, Paisley, UK.
HBSS MEDIUM	GIBCO cell culture; Invitrogen Ltd, Paisley, UK.
HEMATOXYLIN	Sigma-Aldrich Company Ltd. Gillingham, Dorset SP8 4XT, UK.
HYALURONIDASE	Sigma-Aldrich Company Ltd. Gillingham, Dorset SP8 4XT, UK.
L-15 MEDIUM	GIBCO cell culture; Invitrogen Ltd, Paisley, UK.
PERCOLL	Amersham Biosciences Ltd. Buckinghamshire HP7 9NA, UK.
PROPIDIUM IODIDE (1mg/ml)	Sigma-Aldrich Company Ltd. Gillingham, Dorset SP8 4XT, UK.

STREPTOMYCIN/PENICILLIN	GIBCO cell culture; Invitrogen Ltd, Paisley, UK.
TRYPAN BLUE	Sigma-Aldrich Company Ltd. Gillingham, Dorset SP8 4XT, UK.
TRYPSIN (X 10)	GIBCO cell culture; Invitrogen Ltd, Paisley, UK.

8.5.6 DRUGS

DRUG	MANUFACTURER
DOXORUBICIN	Sigma-Aldrich Company Ltd. Gillingham, Dorset SP8 4XT, UK.
MELPHALAN	Sigma-Aldrich Company Ltd. Gillingham, Dorset SP8 4XT, UK.
PACLITAXEL	Sigma-Aldrich Company Ltd. Gillingham, Dorset SP8 4XT, UK.
CISPLATIN	Sigma-Aldrich Company Ltd. Gillingham, Dorset SP8 4XT, UK.
BUSERELIN	Sanofi-Aventis Ltd. Guildford, Surrey GU1 4YS, UK.